

Genetic Studies In Male Breast Cancer

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Abstract

INTRODUCTION

Male breast cancer is a rare disease, accounting for about 0.5% of the total number of breast cancer cases in Scotland. Less is therefore known about the potential genetic influences in its development than for female breast cancer. A number of previous studies have found mutations of the androgen receptor gene and the BRCA2 gene in male breast cancer cases. Polymorphisms in the CYP17, CYP19 and estrogen receptor genes have been associated with an increased risk of female breast cancer.

METHODS

DNA was obtained from 64 male breast cancer cases treated in the Department of Clinical Oncology, Western General Hospital, Edinburgh between 1974 and 1998. The DNA was screened for mutations in the entire BRCA2 gene. Frequencies of polymorphisms in the androgen receptor gene, CYP17, CYP19 and estrogen receptor genes were studied in the 64 cases and in a control population. Family pedigrees for the 64 cases were constructed where possible. Clinical data were retrieved from hospital casenotes.

RESULTS / CONCLUSIONS

Germline BRCA2 mutations were identified in 12 of the 64 (19%) male breast cancer cases. This is an appreciably higher proportion than previously recorded for U.K. populations. A polymorphism in exon 2 of BRCA2 was found significantly more frequently in male breast cancer patients than in controls. The effects of this polymorphism, particularly upon gene expression, require further evaluation. BRCA2 mutations in male breast cancer are not necessarily associated with a positive family history of breast cancer, although half of our series did have affected relatives. In four additional cases, a positive family history was found despite absence of a demonstrable BRCA2 mutation. From the family pedigrees, breast cancer penetrance for the BRCA2 mutations detected as a whole in females by age 73, is estimated at about 62%.

There were no significant differences between the male breast cancer patients found to carry germline BRCA2 mutations compared to those without mutations, in terms of mean age at diagnosis, histological classification of tumours, disease stage at presentation, or survival.

Polymorphic alleles of the CYP17 gene and exon 2 of the estrogen receptor gene are found significantly more frequently in male breast cancer patients than in controls. There were no significant differences in the distribution of alleles of the androgen receptor gene or CYP19 gene between male breast cancer patients and controls.

Chapter 1 - A Historical Review Of Male Breast Cancer

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1.1 The First Recorded Case Of Male Breast Cancer

The first description of an apparent case of male breast cancer was recorded in the Edwin Smith Papyrus. This ancient Egyptian document was written in the seventeenth century B.C. However, it is believed to be a copy of a manuscript written sometime between 2500 and 3000 B.C. It is speculated that Imhotep, chief physician to the pharaoh in the thirtieth century B.C., may have written the original document (Encyclopaedia Britannica Online). The papyrus was acquired in 1862 by an American named Edwin Smith. After Edwin Smith's death in 1906, the papyrus was given to the New York Historical Society by his daughter. In 1930, an American Egyptologist, James Breasted, published a translation. The Edwin Smith Papyrus is organized in a series of 48 surgical cases. A direct copy of the translation of Case 45 is given below (Breasted, 1930). A photograph of the relevant section of the original papyrus is shown in Figure 1.1.

TITLE : Instructions Concerning Bulging Tumors On His Breast

EXAMINATION : If thou examinest a man having tumors on his breast, (and) thou findest that [swellings] have spread over his breast; if thou puttest thy hand upon his breast upon these tumors, (and) thou findest them very cool, there being no fever at all therein when thy hand touches him; they have no granulation, they form no fluid, they do not generate secretions of fluid, and they are bulging to thy hand.

DIAGNOSIS : Thou shouldst say concerning him : "One having tumors. An ailment with which I will contend."

TREATMENT

There is no treatment. If thou findest tumors in any member of a man, thou shalt treat him according to these directions.

As for "Bulging tumors on his breast," it means the existence of swellings on his breast, large, spreading and hard; touching them is like touching a ball of wrappings; the comparison is to a green *hemat*-fruit, which is hard and cool under thy hand, like touching those swellings which are on his breast.

Case 39 in the papyrus describes a swelling over the breast with pus and redness, which felt hot to the touch. It is obvious that the author of the papyrus is making a distinction between this (an infected wound or abscess resulting from an injury) and Case 45, which did not apparently result from an injury.

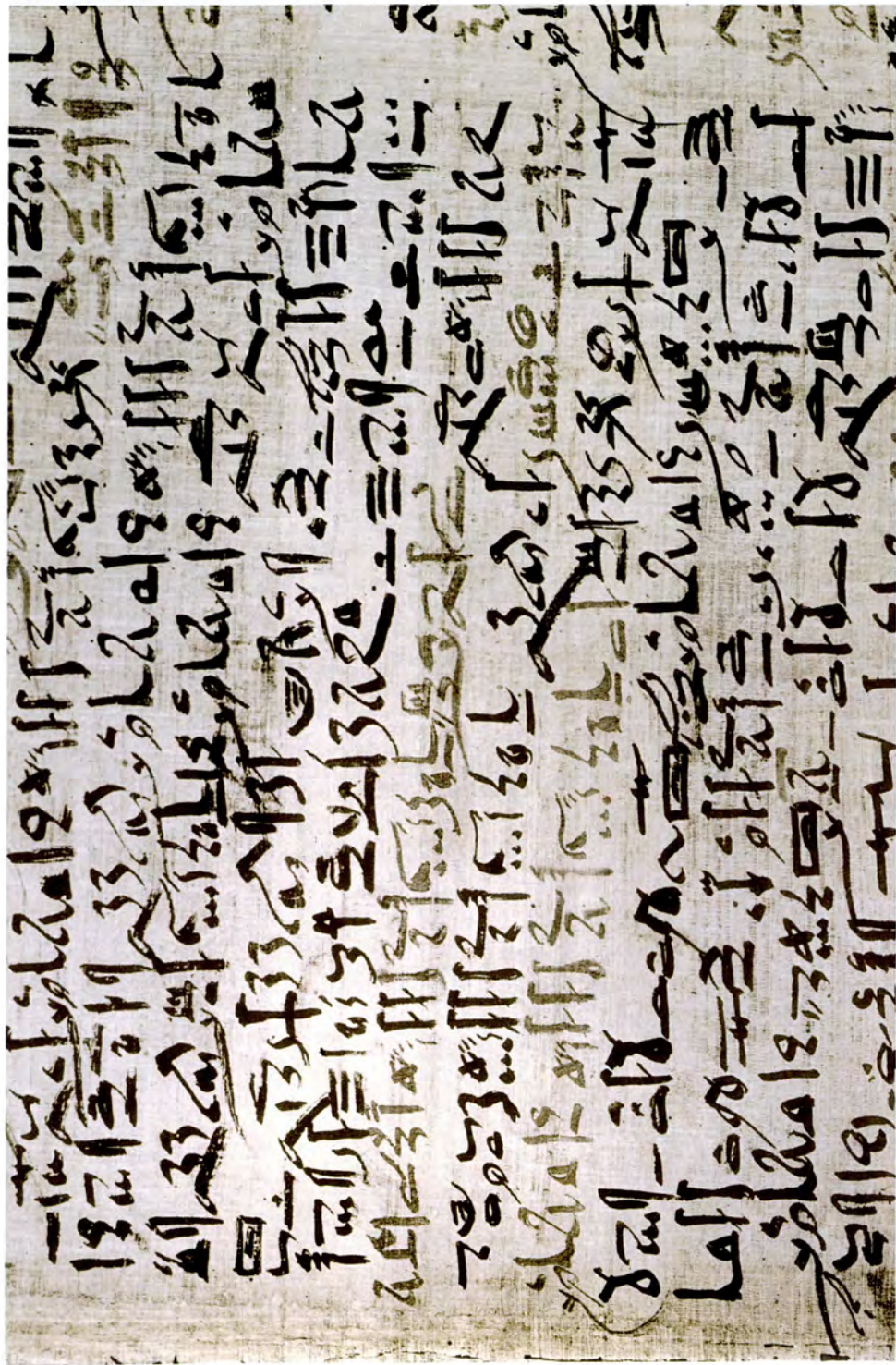
It is perhaps not surprising that the first apparent case of male breast cancer was reported from Egypt. In modern times, the incidence of male breast cancer in comparison to female breast cancer in Egypt is relatively high. El-Gazayerli and Abdel-Aziz (1963) report a consecutive series of 218 cases of breast carcinoma, of which 14 (6.4%) were in males. The authors attribute this high relative incidence of male breast cancer to hyperoestrogenism secondary to bilharzial (Schistosomal) liver fibrosis. There is good evidence that bilharzia (Schistosomiasis) existed in ancient Egypt. Calcified bilharzia ova have been demonstrated on microscopy of the kidneys from mummies (Ruffer, 1910). Evidence of *Schistosoma* infestation has been demonstrated in a predynastic (3200BC) mummy (Deelder et al, 1990).

1.2 Further Historical Records Of Male Breast Cancer

There are several reports of cancer of the male breast published in the Middle Ages (Sasco et al, 1993). The renowned surgeon John of Arderne (c.1307 - c.1390) described a priest who had a 2-year history of a slowly growing wound on the right nipple. Other reports include those by Franciscus Arcaeus (1493-1573) and the father of French Surgery, Ambroise Pare (1510-1590).

Figure 1·1

A photograph of the section of the Edwin Smith papyrus describing Case 45



Chapter 2 - Literature Review

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2.1 Familial Male Breast Cancer

Male breast cancer is a rare disease, accounting for less than 1% of total cases of breast cancer. It is estimated that in the year 2002, 1500 men in the United States will have developed breast cancer, compared with 203500 women (Jemal et al, 2002). Between 1989 and 1998, 156 cases of male breast cancer were registered in Scotland, compared to 32182 cases of breast cancer in women (Scottish Health Statistics, 2001). In other words, 0.48 % of cases of breast cancer registered during this period in Scotland, were in men.

Because of the rarity of male breast cancer, most reported series involve relatively small numbers of cases. Less is therefore known about the potential genetic influences in its development than for female breast cancer.

The possibility of a familial component to the aetiology of male breast cancer has been recognized for many years. In 1889, Williams gave a record of 100 cases of cancer of the male breast. Inquiries about family history had been made in 29 of these cases, and there was a history of cancer in seven families. In one of these families, the patient's father was said to have had breast cancer, although no further details are given as to whether this was verified. In two of the families, the patient's sister had breast cancer and in another family a maternal aunt was affected.

In 1926, Judd and Morse described 17 cases of male breast cancer presenting to the Mayo Foundation. The mother of one of these cases died with carcinoma of the breast and three other cases gave a family history of cancer.

Schottenfeld et al (1963) reviewed 120 male breast cancer patients treated throughout the U.S. between 1955 and 1962. 53 of these patients were interviewed and two reported breast cancer in another male family member.

Marger et al (1975) reported the first well-documented cases of breast cancer occurring in male relatives. These authors reported 30 cases of male breast cancer presenting to a single hospital over a 50-year period. Two of the cases, aged 52 and 69 at the time of diagnosis, were brothers. The elder of the two had also been diagnosed with adenocarcinoma of the prostate three years previously and had been treated with oestrogen. The sister of another of the cases had bilateral breast cancer. At the time of this publication, it was already known that the risk of breast cancer in a woman was increased if her mother had been affected. The authors suggested that it might be worthwhile looking at the incidence of breast cancer in women with affected fathers, to see if an increased risk was conferred to them in this manner.

In 1976, Everson et al described the occurrence of male breast cancer in two families. In one family, three brothers and four female relatives on the maternal side had breast cancer. In the other family, the proband, his father and a paternal uncle had breast cancer. Within both of these families there was no consanguinity and there was nothing to suggest occupational exposure to any possible aetiological agents. The occurrence of breast cancer in both males and females within one family, led the authors to suggest that familial susceptibility to breast cancer can occur regardless of gender. Increased urinary excretion of oestrogen was found in one of the males with breast cancer and also two unaffected male relatives of cases. It was therefore suggested that in these patients there might have been an abnormality in oestrogen production or metabolism.

Teasdale et al (1976), report a male who developed breast cancer aged 59 and gastric cancer aged 66. His brother developed breast cancer aged 70, and his daughter aged only 26. It was suggested that paternal genetic factors may have increased the

susceptibility of the proband's daughter to breast cancer. Interestingly, a sister of the proband had ovarian cancer and another brother had oral cancer.

Several more recent studies have now demonstrated, and attempted to quantify, an increased risk of breast cancer among female relatives of breast cancer cases, with a relative risk varying between two and sixteen times the population risk (Anderson & Badzioch, 1992; Storm & Olsen 1999).

While familial male breast cancer has been recognized for more than a hundred years, it is only within the last decade that responsible genetic factors have been postulated. These are discussed below.

2.2 Androgen Receptor Gene

The first report of a germline genetic mutation linked to male breast cancer was in the androgen receptor gene within codon 607 (Wooster et al, 1992). This mutation was found in two brothers who both had androgen insensitivity and developed breast cancer aged 55 and 75 years. It was thought possible that the development of breast cancer in these men may have been due to abnormal stimulation of the breast epithelium, with loss of the protective effect of androgens. Alternatively, the androgen receptor gene mutation itself may have had specific biological effects that increased breast cancer risk. Another mutation in the androgen receptor gene within codon 608 has since been reported in an isolated male with breast cancer and partial androgen insensitivity (Lobaccaro et al, 1993). This mutation resulted in an amino acid substitution adjacent to the first reported mutation, suggesting that the association between male breast cancer and mutations in the androgen receptor gene is not purely coincidental. On the other hand, Haraldsson et al (1998) in a study of 34

male breast cancer cases from Sweden, found no evidence of germline or somatic androgen receptor gene mutations.

Another study also failed to identify any somatic mutations within the androgen receptor gene in breast cancers (Shan et al, 2000). However, somatic reductions in the length of a CAG repeat sequence within the androgen receptor gene have been found in colonic cancers (Ferro et al, 2000).

2.3 BRCA1

The breast cancer susceptibility gene BRCA1 was mapped to chromosome 17q (Hall et al, 1990 & Narod et al, 1991). It was cloned in 1994 (Miki et al). Familial and early onset female breast cancer have been linked to mutations in the BRCA1 gene (Futreal et al, 1994; Castilla et al, 1994; Friedman et al, 1994; Gayther et al, 1995). However, subsequent studies described below suggest that the association between BRCA1 mutations and male breast cancer is much less strong.

Stratton et al (1994) reported a study of 22 families, all with at least one case of male breast cancer. In this study, linkage analysis was performed and it was estimated that 0% (95% C.I. 0% to 18%) of the families were linked to BRCA1. The authors suggested that probably only a small proportion of breast cancer families containing a male case are linked to BRCA1, and that there is another gene associated with a higher risk of male breast cancer. In a large study of 54 male breast cancer cases from Southern California (Friedman et al, 1997), no mutations were found in BRCA1.

Isolated cases of male breast cancer have, however, been reported in carriers of BRCA1 mutations. Hogervorst et al (1995) detected a BRCA1 mutation within a

family containing a male breast cancer case, however no DNA was available from the affected male for testing. Struwing et al (1995) studied 24 families, each with at least one case of ovarian cancer and a total of at least three cases of breast and ovarian cancer. Eight BRCA1 mutations were detected in ten families. One of these families contained a male affected by breast and prostate cancer, who was found to carry a BRCA1 mutation. In a large study of 237 families, Ford et al (1998) found BRCA1 mutations in two families from 14 screened, that contained a case of male breast cancer. However, it was not stated whether the affected males themselves, or just other affected female family members were screened. In another large study from Israel, a BRCA1 founder mutation was detected in four males with breast cancer, out of 124 males that were screened (Struwing et al, 1999). However, approximately 1% of the Ashkenazi Jewish population as a whole are carriers of this mutation (Fodor et al, 1998).

2.4 BRCA2

In 1994, Wooster et al localized the BRCA2 gene to chromosome 13q. This study included six families containing cases of male breast cancer. A study from Iceland (Thorlacius et al, 1995) linked a family with four cases of male breast cancer to the BRCA2 gene. The BRCA2 gene has subsequently been identified (Wooster et al 1995). It is made up of 27 exons (the first of which is non-coding) and it encodes a protein made up of 3418 amino acids (Tavtigian et al, 1996).

Several possible functions of BRCA2 have been suggested. BRCA2 may have a role in the regulation of transcription, and certainly, one study has demonstrated that the BRCA2 protein could stimulate transcription (Milner et al, 1997).

Experimental studies in mice have shown that cells without a functional BRCA2 gene, when subjected to irradiation, fail to efficiently repair double-strand DNA breaks (Connor et al, 1997). In cells that do not have a functioning BRCA2 gene, there is a spontaneous instability of chromosome structure and abnormalities of chromosome number (Patel et al, 1998). Many of the chromosomal abnormalities develop during DNA replication. Non-homologous repair of DNA breaks (by simply joining the broken ends of DNA) can occur without a functional BRCA2 gene. However this type of repair is prone to inducing errors at the site of repair (Yu et al, 2000). A role for BRCA2 in the homology-directed repair of double-strand breaks in DNA has been demonstrated (Moynahan et al, 2001). In other words, BRCA2-deficient cells are unable to repair DNA by homologous recombination, which involves exchanging the broken DNA for a perfect copy, and is therefore error-free. The consequence is that there is increased chromosomal instability within these cells. Homologous recombination involves a protein called RAD51 that is involved in the repair of double-strand breaks in DNA and also in mitotic and meiotic recombination (Sharan et al, 1997). The BRCA2 protein sequence contains eight repetitive units known as BRC motifs (Bork et al, 1996; Bignell et al, 1997). Binding sites for RAD51 have been mapped to the BRC motifs in BRCA2 (Wong et al, 1997; Chen et al, 1998). This suggests that BRCA2 regulates homologous recombination through its interaction with RAD51 (Venkitaraman, 2002). More recent structural studies have shown that a small molecule called Dss1 binds to the carboxy-terminal end of BRCA2 (Yang et al, 2002). The BRCA2-Dss1 complex can bind to single- and double-stranded DNA. The ability of this complex to bind to DNA suggests that BRCA2 may carry RAD51 to sites of DNA damage, ultimately leading to DNA

repair (Venkitaraman, 2003). It has also been shown that polymorphisms in RAD51 increase the risk of breast cancer among carriers of BRCA2 mutations (Levy-Lahad et al, 2001; Wang et al, 2001).

Wooster et al (1995) also reported the first BRCA2 mutations to be found in male breast cancer cases. The published detected frequency of BRCA2 mutations among groups of male breast cancer patients varies considerably between different populations. In a series of 54 male breast cancer cases from Southern California, only two (4%) were found to have germline BRCA2 mutations (Friedman et al, 1997). This contrasts with the 13 cases from a series of 34 (38%) in Iceland (Thorlacius et al, 1998). However, all of these 13 cases carried a single founder BRCA2 mutation thought to originate from a single common ancestor at least 500 years ago. There have now been many published series including male breast cancer cases in which BRCA2 mutations have been detected (Table 2.1).

In addition to male and female breast cancer, germline mutations in BRCA2 are well described in familial ovarian carcinoma (Wooster et al, 1995; Tavtigian et al, 1996; Easton et al, 1997; Schubert et al, 1997; Gayther et al, 1997). They have also been found, albeit more infrequently, in sporadic ovarian carcinomas (Takahashi et al, 1996). Germline BRCA2 mutations have also been associated with a variety of other cancers, including prostate (Easton et al, 1997; Struwing et al, 1997; Breast Cancer Linkage Consortium, 1999; Gayther et al, 2000) and pancreas (Phelan et al, 1996; Goggins et al, 1996; Breast Cancer Linkage Consortium, 1999; Grönberg et al, 2001; Murphy et al, 2002).

Table 2·1

Published series including male breast cancer cases in which BRCA2 mutations have been detected

Series	Country	No. MBC Cases	No. BRCA2 Mutations
Couch et al (1996)	U.S.A.	50	7 (14%)
Friedman et al (1997)	U.S.A.	54	2 (4%)
Mavraki et al (1997)	England	28	3 (11%)
Thorlacius et al (1998)	Iceland	34	13 (38%)
Haraldsson et al (1998)	Sweden	34	7 (21%)
Csokay et al (1999)	Hungary	18	6 (33%)
Struewing et al (1999)	Israel	124	15 (12%)
Diez et al (2000)	Spain	17	3 (18%)
Wolpert et al (2000)	Canada	14	2 (14%)
Kwiatkowska et al (2001)	Poland	37	4 (11%)
Pages et al (2001)	France	12	4 (33%)
Basham et al (2002)	England	94	5 (5%)
Ottini et al (2003)	Italy	25	3 (12%)

Whilst germline mutations in BRCA2 are well described, somatic BRCA2 mutations in breast and ovarian carcinomas are relatively rare (Lancaster et al, 1996; Miki et al, 1996; Takahashi et al, 1996; Weber et al, 1996; Foster et al, 1996). There is only one report in which a somatic BRCA2 mutation was detected in a single male breast cancer case (Kwiatkowska et al, 2002).

2.5 Other Genes

Germline p53 mutations have been reported in a few cases of female breast cancer associated with Li-Fraumeni syndrome (Rapakko et al, 2001). To date there are no reports of germline p53 mutations in male breast cancer cases. However, somatic p53 mutations are common in carcinomas of the male breast (Anelli et al, 1995).

A germline mutation of the MLH1 mismatch repair gene has been described in a male who developed colonic carcinoma aged 41 and breast cancer aged 72 (Boyd et al, 1999). He was a member of a hereditary non-polyposis colorectal cancer (HNPCC) family. Another study (Borg et al, 2000) describes two HNPCC families that include male and female breast cancer cases. Both had germline mutations in MLH1, but one male also had a mutation in BRCA1.

Cowden syndrome is associated with mutations in the PTEN (phosphatase and tensin homologue mutated on chromosome ten) tumour suppressor gene. A recent study describes germline mutations in the PTEN gene in two families containing cases of male and female breast cancer (Fackenthal et al, 2001).

The gene CHEK2 (otherwise known as CHK2) codes for a kinase that is activated as a response to DNA damage (Matsuoka et al, 1998; Chaturvedi et al, 1999). Germline CHEK2 variants have previously been reported in Li-Fraumeni families where no

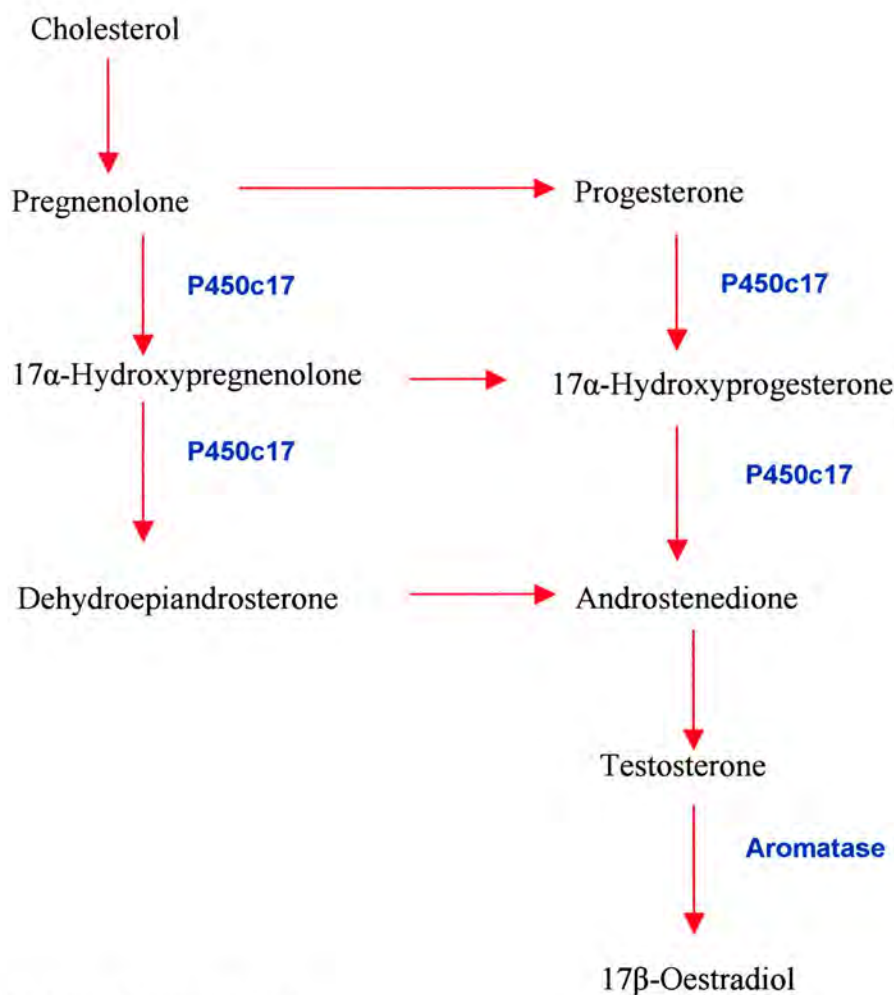
p53 mutations were found (Bell et al, 1999). In one recent study (Meijers-Heijboer et al, 2002), a variant in CHEK2 (1100delC) was found in 55 out of 1071 (5.1%) families with breast cancer in whom no BRCA1 or BRCA2 mutations had been found. This was a higher than the 1.1% of healthy controls found to carry the CHEK2 variant. This study also found the variant in 7 out of 52 (13.5%) families that contained at least one case of male breast cancer. The authors concluded that within BRCA1 and BRCA2 negative families, this variant gave a relative risk for female breast cancer of approximately two and for male breast cancer, an approximately ten times increase in risk. A subsequent study has also reported an association between the CHEK2 1100delC variant and familial female breast cancer (Vahteristo et al, 2002).

Mutations of the genes discussed above clearly confer a greatly increased risk of breast cancer on a carrier. However, the frequency of such genetic mutations within whole populations is relatively small. A recent meta-analysis showed an increased relative risk of breast cancer of 2.3 (95% confidence interval 1.6-3.2) associated with high levels of oestrogens in post-menopausal women (Key and Verkasalo, 1999). Male breast cancer is also known to be associated with increased levels of serum oestrogens, for example, in Klinefelter's syndrome (Jackson et al 1965). Any genetic changes that may result in elevation of the level of oestrogens may therefore result in an increased risk of developing breast cancer. The risk to an individual conferred by high oestrogen levels is estimated at 2.3 times greater than the general population risk (as indicated above). The risk of female breast cancer in the Scottish population as a whole is reported to be 7.9% to the age of 74 (Cancer Registration Statistics Scotland 1986 – 1995). The risk to those individuals with high oestrogen levels

would therefore be approximately 18%. This is lower than the cumulative risk to age 70 associated with carriage of a BRCA1 or BRCA2 mutation, which is estimated to be between 26% and 56% by population-based studies (Satagopan et al, 2001; Struewing et al, 1997). However, in general population terms, oestrogens are likely to be important, given that the carriage of a germline mutation is rare. The pathway for synthesis of androgens and oestrogens is shown in Figure 2.1.

Figure 2.1

Pathway for the synthesis of androgens and oestrogens



(Adapted from Ganong, 1993)

The CYP17 gene on chromosome 10 codes for the Cytochrome P450c17 enzyme that catalyses steroid 17 α -hydroxylase and 17,20-lyase activities (Picado-Leonard, 1987). It is therefore a key regulator in the synthesis of androgens and oestrogens from their steroid precursors (Brentano et al, 1990).

A polymorphic T \rightarrow C substitution has been described that creates an additional CCACC type promoter site 34bp upstream from the site of initiation of translation (Carey et al, 1994). It is thought that the additional promoter site may increase the rate of transcription of the gene and thereby increase enzyme activity. This polymorphism also generates a restriction site for the enzyme MspA1. Serum oestradiol levels are higher in women hetero- and homozygous for the C allele of the CYP17 gene (Feigelson et al, 1998). One study found that the C allele was associated with an increased risk of advanced breast cancer (Feigelson et al, 1997). A larger and more recent study, however, found no such association (Dunning et al, 1998). Given that the C allele of the CYP17 gene is associated with increased serum oestradiol levels, then it could also be implicated in the development of male breast cancer. The association with advanced breast cancer in females may also be relevant to males, given that men with breast cancer tend to present with more advanced disease than women (Ravandi-Kashani & Hayes, 1998).

The CYP19 gene (located on chromosome 15q21.1) codes for the aromatase enzyme that controls the rate limiting step in the pathway of oestrogen synthesis from steroid precursors (Figure 2.1). As already stated, it is known that an increased risk of breast cancer in males is associated with elevated serum oestrogen levels. The aromatase enzyme has been observed within the stromal cells in a greater proportion of male

breast carcinomas than gynaecomastia cases, suggesting that locally produced oestrogens may also have a significant role in the development of male breast cancer (Sasano et al, 1996). It is therefore possible that variation in expression of the CYP19 gene could affect the risk of developing male breast cancer.

A polymorphic tetranucleotide (TTTA) repeat sequence is found in intron 5 of the CYP19 gene, 79 nucleotides downstream from exon 4 (Means et al, 1989). This repeat sequence is relatively close to the exon / intron border and may therefore be involved in the determination of splicing sites (Kristensen et al, 1998).

A region within exon 1 of the gene coding for the androgen receptor (located on chromosome Xq11-12) is highly polymorphic and contains a variable number of CAG repeats. The variability of the number of these repeats between different ethnic populations in the U.S. has been studied (Edwards et al, 1992). *In vitro* studies have shown that a relatively short CAG repeat sequence increases the level of transactivation of the androgen receptor (Chamberlain et al, 1994). The androgen receptor itself binds dihydrotestosterone and therefore is one factor in the regulation of the growth of prostate cells. This may account for the finding that short CAG repeat sequences have been associated with a higher risk of developing prostate cancer (Stanford et al, 1997; Giovannucci et al, 1997). Abnormally long sequences of 40 repeats or more are found in patients with X-linked spinal and bulbar muscular atrophy, otherwise known as Kennedy's disease (LaSpada et al, 1991). This disease is associated with gynaecomastia and reduced fertility, indicating androgen insensitivity. Mutations of the androgen receptor gene, found in a few cases of male breast cancer as described above, may also result in reduced androgen receptor

function. It is therefore possible that long CAG repeat sequences in the androgen receptor gene are associated with an increased risk of male breast cancer.

The human oestrogen receptor (ESR1) gene is located on chromosome 6q24-q27.

Other published studies have looked at polymorphisms in the oestrogen receptor gene, but have not shown a consistent association with female breast cancer

(Andersen et al, 1994; Schubert et al, 1999; Yaich et al, 1992). One of these

polymorphisms is a T→C substitution in Intron 1. This single-base change results in

loss of a restriction site for the enzyme PvuII. Another of the polymorphisms in the

oestrogen receptor gene is a G→C substitution in Exon 2. This generates a restriction site for the enzyme BstUI.

2.6 Hypotheses And Aims Of The Study

The original hypotheses prior to commencing the study were :

1. BRCA2 mutations might be commoner among male breast cancer cases in Scotland than elsewhere in UK.
2. There might be founder BRCA2 mutations in Scotland.
3. There might be an excess of other cancers among the male breast cancer cases and their relatives.
4. A polymorphism in the CYP17 gene might increase the risk of male breast cancer.

The aims of the study were as follows :

1. To screen a large series of male breast cancer patients for mutations in the BRCA2 gene.
2. To construct detailed family pedigrees for these patients, and hence to evaluate male breast cancer as a risk factor for female breast cancer in relatives.
3. To determine whether or not the clinical outcome is different for male breast cancer that is associated with BRCA2 mutations.
4. To establish whether the C allele of the CYP17 gene is associated with an increased risk of male breast cancer.
5. To determine whether the development of male breast cancer is influenced by the length of a tetranucleotide repeat sequence in the CYP19 gene.
6. To investigate whether increased length of the CAG repeat sequence in the androgen receptor gene is associated with the development of male breast cancer.
7. To investigate the frequencies of two oestrogen receptor gene polymorphisms in male breast cancer cases.

Note : The aims numbered 5 to 7 were only conceived after the study commenced and were therefore not part of the original hypothesis.

Chapter 3 – Review Of Laboratory Methods

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3.1 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is an *in vitro* method for the amplification of a segment of DNA (Mullis et al, 1986). This allows an adequate quantity of a specific sequence of DNA to be produced for screening to identify mutations and polymorphisms. Changes in temperature are needed for this *in vitro* reaction to occur. Firstly, by heating to about 94°C, the bonds holding the strands together can be broken so that the DNA is single-stranded. The temperature of the reaction is then dropped to between 50 and 60°C. Two oligonucleotide primers can then hybridise to complementary strands of the DNA, flanking the region of interest. The new DNA is synthesised on to the 3'-end of the two primers by a heat-stable DNA polymerase, usually at 72°C. This process is then repeated. In theory, the amount of specific sequence DNA doubles each cycle. In reality, if the reaction is performed 30 times, then about 10^5 copies of the specific sequence should be made (Strachan & Read, 1997). This process would take only a few hours. The process of temperature cycling is automated using thermocyclers, which are sophisticated programmable hot blocks. Screening of genomic DNA for mutations can be performed once adequate quantities of the specific sequence to be studied have been produced by PCR. The gold-standard method of identifying mutations is DNA sequencing. However this is very expensive if entire genes are to be screened. Methods that detect possible sequence changes, and allow targeted sequencing are therefore much more cost-effective. The methods that have been used for screening the BRCA2 gene in our study are reviewed below.

3.2 Heteroduplex Analysis

Formation of DNA complexes called heteroduplexes can occur when two DNA strands that differ in their sequence anneal together. This was first described by Nagamine et al (1989). In this study, one of the strands contained a deletion of 18 bases. It was hypothesised that either the strands might be annealed with the extra 18 bases on one strand forming a bulge, or the strands might only be annealed on the 3' side of the deletion. The consequential difference in secondary structure resulted in abnormal migration of the heteroduplex structure through a polyacrylamide gel. It has since been suggested that a more marked conformational change in the structure of the DNA, a bend, would produce a greater difference in migration between a homoduplex structure and a heteroduplex one (Ganguly et al, 1993). Another study showed that a smaller deletion of 3-bp in the cystic fibrosis gene could also be detected using this method (Rommens et al, 1990). However, it is possible to detect even more subtle genetic alterations, i.e. single base pair substitutions, by the process of heteroduplex analysis (HA) (Keen et al, 1991). The appearance of heteroduplexes after electrophoresis on a gel are illustrated in Figure 3.1.

HA has been shown to be a sensitive technique in the detection of single base-pair substitutions. In one study, eight out of nine (89%) single base substitutions were detected (White et al, 1992). The overall sensitivity of HA in detecting sequence changes has been reported as low as 60% (Choy et al, 1999), but in other studies as high as 92% (Körkkö et al, 2002) and 100% (Körkkö et al, 1998). The specificity of HA is uniformly reported to be 100%. Any errors within the screening process are most likely to arise during PCR rather than the subsequent analysis of PCR products. The advantages of HA are that it is a relatively simple process, there is no need for

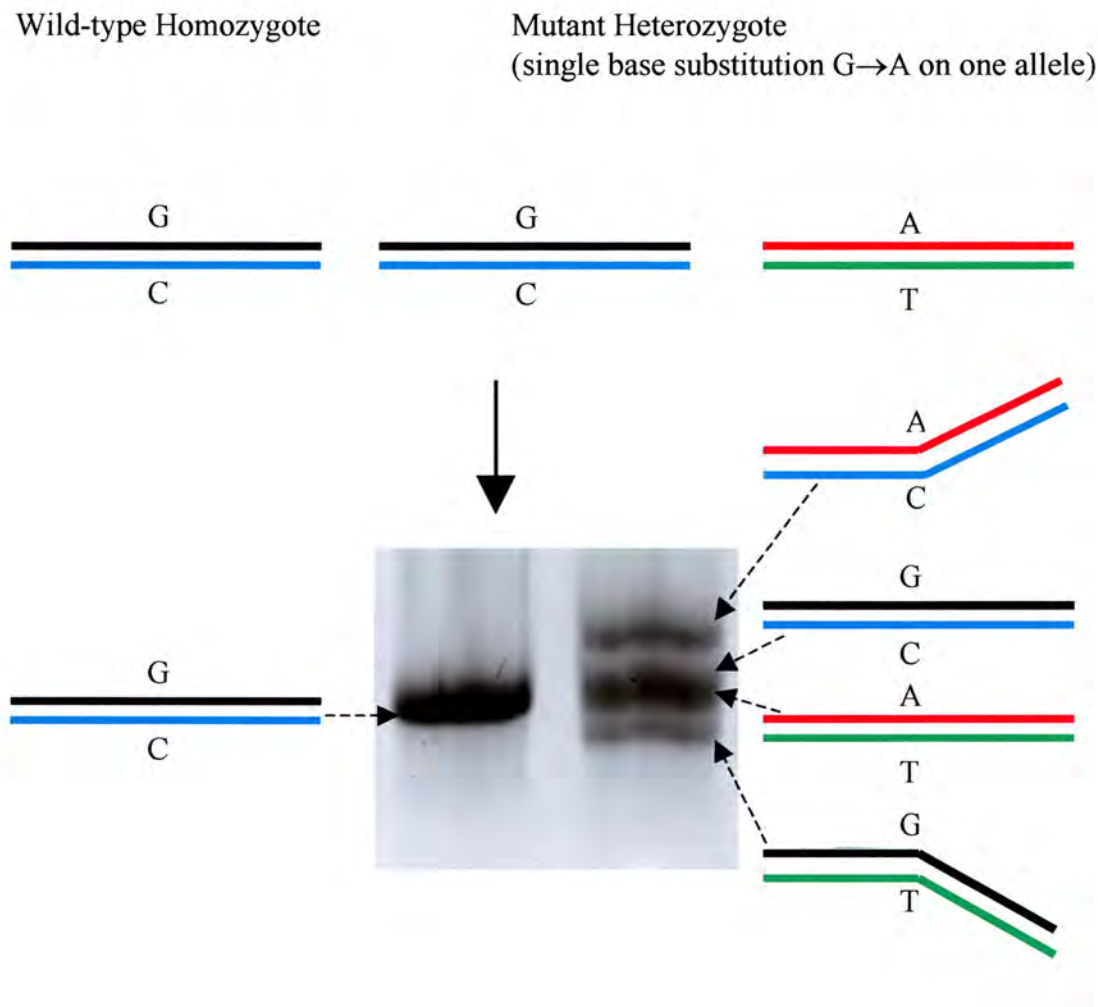
specialist equipment or special preparation of the PCR product and it is not necessary to use radioactivity in order to visualise the bands on the gel. The limitations of HA for mutation detection include the requirement for subsequent sequencing of PCR products found to contain abnormalities, a large number of which may be insignificant polymorphisms. HA also gives no indication as to the position of the sequence change within the PCR product. The length of the PCR product under study appears to be important and is one of the factors that affect the sensitivity of HA. It has been shown that the optimal length of the PCR product should be between 150 and 200bp (Ganguly et al, 2002).

An alternative method of screening PCR products for sequence changes is single-strand conformation polymorphism (SSCP). This process depends upon the single strands of denatured DNA forming different conformations if their sequences differ. The sensitivity of SSCP appears to be highly dependent upon the size of the DNA fragments analysed. For fragments of 150-212bp in size, the sensitivity for detection of single base substitutions in one study was 79% (Sheffield et al, 1993). In this study, sensitivity was lower for larger fragments and for fragments less than 150bp in length. Several studies have compared heteroduplex analysis directly with SSCP. In one study, SSCP detected only seven of the eleven mutations detected by HA (Henderson et al, 1997). However, in another direct comparison between the two methods the sensitivity was similar, 85% for SSCP and 82% for HA (Jones et al, 1999). In another study (Rosetti et al, 1995), the sensitivity for HA was found to be 81% and SSCP 69%. Importantly however, for fragments between 257 and 426bp in length, the sensitivity of HA was 100%, but SSCP only 20%. For the screening of BRCA2 in our study, 44 of the 62 primer pairs produce PCR products within this

size range. Using this set of primers, HA is therefore likely to be a more sensitive method of screening than SSCP.

Figure 3-1

An illustration of the appearance of heteroduplexes after electrophoresis on a gel



HA is an effective method of screening a group of samples for a single mutation, however if the entire coding sequence of a gene is screened, then the process can be laborious. Several different areas of the gene can be screened at once, if the PCR products are of different length, by use of a multiplex analysis (Gayther et al, 1996).

Recently, more rapid automated methods of performing HA have been developed (Tian et al, 2000; Edwards et al, 2001).

3.3 Protein Truncation Testing

Just over 50% of reported mutations in BRCA2 are frameshift or non-sense (Breast Cancer Information Core), and therefore might be expected to result in premature termination of translation and consequent truncation of the protein product. Some techniques that have been used to detect DNA sequence changes (such as HA, described above) may identify polymorphisms and mis-sense sequence changes which are of doubtful significance. A technique that is able to detect mutations that result in a truncated protein product alone would therefore be a valuable tool, if rapid screening of a gene were required. While avoiding the detection of possibly meaningless mis-sense changes, this method may however fail to detect truncating mutations at either end of the exon (Davies et al, 2000).

Such a technique, known as the protein truncation test (PTT), was initially developed to screen the Duchenne Muscular Dystrophy (DMD) gene (Roest et al, 1993) and the APC gene (Powell et al, 1993; van der Luijt et al, 1994). More recently, PTT has been used for the detection of mutations in BRCA1 (Hogervorst et al, 1995; Plummer et al, 1995) and BRCA2 (Garvin, 1998).

The coding region of a gene is screened for mutations that result in premature termination of translation, using protein that is synthesised *in vitro*. First, an amplified DNA product corresponding to the coding sequence of the gene to be

studied is obtained. This is done either by PCR amplification of genomic DNA, or by reverse transcription PCR (RT-PCR) amplification of mRNA.

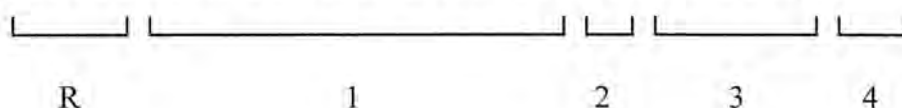
For each PCR used during PTT, a special sense / forward primer with an additional 'tail' is designed (Hogervorst, 1997). This primer contains four regions, three of which form the 'tail'. Firstly, a T7 promoter sequence is at the 5'-end of the primer. This directs the production of RNA using an RNA Polymerase. There is then a spacer sequence of 3-6bp in length. Next is a eukaryotic translation initiation sequence, otherwise known as the Kozak sequence (Kozak, 1984). The 3'-end of the primer contains the target gene sequence (approximately 17-20bp in length). A restriction site sequence is sometimes incorporated into the 5'-end of the primer. This allows cloning of the amplified product. PTT primers previously used for screening of exons 10,11 and 27 of BRCA2 (Lancaster et al, 1996) contained the T7/Kozak sequence shown in Figure 3.2.

The DNA sequence of the tailed forward primer clearly is not identical to the sequence of the genomic DNA under study and there may therefore be problems in terms of the primer not annealing. In order to overcome this potential problem and to increase the yield of PCR product, nested PCR can be performed. This involves an initial PCR amplification of the genomic DNA using standard primers. A secondary PCR reaction is then performed using the PTT primer as the forward primer and the primary PCR product as the DNA template.

Figure 3.2

Components of the forward primer used for PTT

5'- GGATCCTAATACGACTCACTATAGGGAGACCACCATG..... -3'



- | | | | |
|---|---------------------------|---|------------------------------------|
| R | Restriction Site Sequence | 1 | Bacteriophage Promoter T7 Sequence |
| 2 | Spacer | 3 | Kozak Sequence |
| 4 | Target gene sequence | | |

The PCR products that are produced are checked by electrophoresis on agarose gels. If products of abnormal size are seen, then this can indicate the presence of deletions or duplications. If mRNA has been used, then mutations within splicing sites may result in a PCR product of abnormal length. The PCR products are then used for the *in vitro* synthesis of RNA. This RNA is then translated into protein. This process of *in vitro* transcription followed by translation, has been greatly simplified by development of the TnT® Quick Coupled Transcription/Translation System (Promega).

In order to determine whether or not a truncated protein product has been synthesised, the proteins need to be separated, according to their size, by electrophoresis. The disulphide bridges in proteins need to be reduced before they can adopt the necessary configuration to allow separation by electrophoresis. This can be done using β -mercaptoethanol (Strachan & Read, 1997). Sodium dodecyl sulphate (SDS) binds to proteins and gives them a negative charge. The proteins

undergo denaturing SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE). The distance that the proteins migrate down the gel is inversely related to their molecular weight.

The proteins can be visualised by the incorporation of radio-labelled amino acids when they are being synthesised. Non-radioactive methods of protein detection have been developed, for example the Transcend[™] Colorimetric Translation Detection System (Promega). This involves the incorporation of biotinylated lysine residues into the protein. The biotinylated proteins can be visualised by binding Streptavidin-Alkaline Phosphatase followed by Western Blue[®] Substrate to produce coloured bands.

Chapter 4 – Methods

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Ethical approval for the study was obtained from the Lothian Regional Ethics committee.

4.1 Male Breast Cancer Cases

The initial basis for our study came from a clinical review that had been performed of 55 male breast cancer patients treated in the Department of Clinical Oncology, Western General Hospital, Edinburgh between 1974 and 1993 (Andrews et al, 1996). This list of 55 cases was updated to include those treated between 1994 and 1998. There were then a total of 76 male breast cancer patients, treated over a 25-year period. A summary of how DNA samples were obtained from the male breast cancer cases is shown in Figure 4.1.

26 of the 76 male breast cancer cases were alive in 1998. Contact was made by telephone with the General Practitioners of these patients to discuss the study. In each case, permission was sought from them to approach the patient concerned. Initial contact was then made with the patient by telephone, or by letter if this was not possible. An interview with each patient was then arranged. Only two patients declined to take part in the study. The study was discussed with the patient and written consent was obtained. A detailed family history was then taken from each patient and recorded on the form shown in Appendix 7.1. 10ml peripheral blood samples were obtained from each of the patients in four 2.5ml tubes containing EDTA and frozen at -70°C as soon as possible thereafter.

For the deceased patients, details of the hospital where a biopsy was taken or surgical treatment was performed were obtained from the hospital casenotes (stored in the Department of Clinical Oncology). Pathology specimen numbers were retrieved

where recorded. A pathologist in the hospital concerned was then contacted and archival wax-embedded tissue sections were obtained where possible. Archival tissue samples were obtained for 39 deceased patients, as detailed in Figure 4.1. No specimens obtained at post-mortem examination were used. DNA previously extracted from a blood sample and stored was available for one of the deceased patients. Archival specimens were not available in 10 cases.

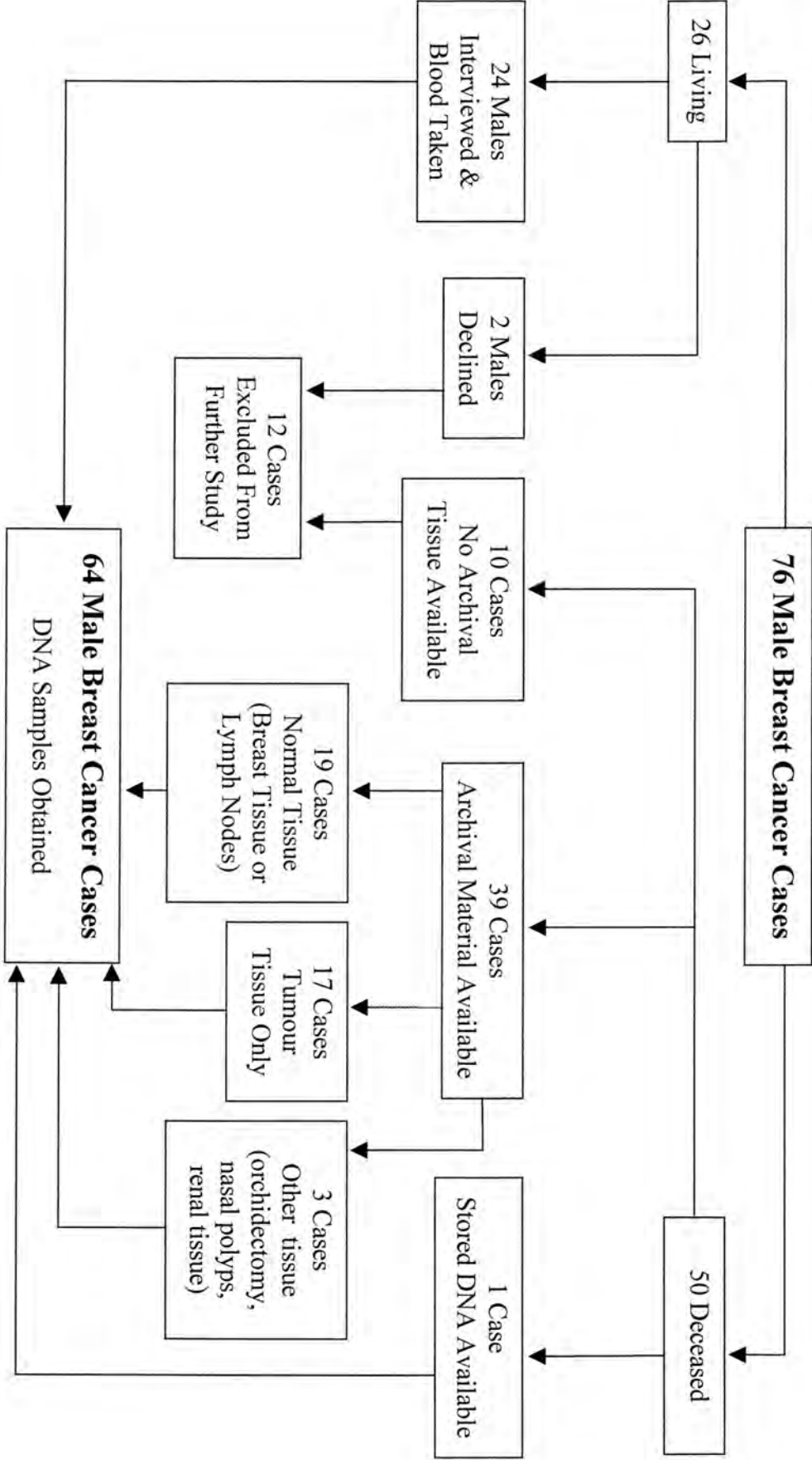
For analysis of a polymorphism in the CYP17 gene, female breast cancer cases were also required. These were from blood samples taken from a consecutive group of 39 patients with early onset (age <50 years) breast cancer referred to the Edinburgh Breast Unit, Western General Hospital, Edinburgh between 1987 and 1990.

4.2 Control Population Selection

Male and female control DNA samples were extracted from blood donations to the Edinburgh and South East Scotland Blood Transfusion service (82 samples), from placental tissue and blood donations to the School of Biological and Medical Sciences, University of St. Andrews (49 samples), and from tonsillectomy specimens provided by the Department of E.N.T. Surgery, City Hospital, Edinburgh (8 samples). In order to ensure confidentiality, the only information that we were given about the original donors was their gender. These controls were not matched for age with the cases, however the majority were from blood donors, thereby including healthy members of the general population between the ages of 17 and 70. We believe that these samples were as representative of the population within South East Scotland as was possible to obtain.

Figure 4-1

How DNA samples were obtained from the male breast cancer cases



4.3 Family Pedigrees

For all Scottish-born patients (living or deceased) a complete family tree extending for at least three generations on both maternal and paternal sides was constructed. This was performed by an experienced genealogist (Alison Fordyce) by examining the Scottish public records of births, deaths and marriages (held by the Registrar General for Scotland). The use of the Scottish Public Records in similar situations is described by Collyer and DeMey (1987). In Scotland, the actual records of births, deaths and marriages can be viewed directly. In England, a record has to be purchased before it can be viewed. For this reason, and because of the larger population of England, it is much easier to construct pedigrees in Scotland. The information and statistics division (ISD) of the Scottish NHS Common Services Agency is responsible for the statistical analysis of all health service records. Their databases include all hospital discharge records and population based cancer registers. The cancer registration system includes cases confirmed histologically (approximately 80%) and has data of good quality from 1968 to date. The names and dates of birth of relatives (parents, grandparents, siblings, uncles, aunts and cousins) of the male breast cancer cases were extracted from the pedigrees. The Scottish Cancer Registry was examined to see if any of these individuals had a diagnosis of fatal or non-fatal cancer. For reasons of confidentiality, the Cancer Registry would only provide such information on relatives who are now deceased. For the male breast cancer cases who were deceased prior to May 2002, dates of death were provided by the cancer registry.

4.4 Clinical Data

Clinical data for the patients were retrieved from hospital case records. Details for each male breast cancer case in terms of age at diagnosis, disease stage at presentation, histological type, and loco-regional disease recurrence were recorded. For survival, the length of time between date of diagnosis (obtained from the case records) and either the date of death (from the cancer registry data), or May 2002 (if the patient is still alive) was calculated. The patients subsequently found to have germline mutations in BRCA2 were grouped together and compared to those not found to carry mutations. The clinical data were then analysed with the help of a statistician (Gill Kerr).

4.5 DNA Extraction From Whole Blood

Within a Class I microbiological safety cabinet, 5mls of whole blood were lysed in a 50ml polypropylene centrifuge tube with 5mls of blood lysis buffer (0.1M Tris pH 8.0, 20mM NaCl, 1mM Ethylenediaminetetraacetic acid (EDTA), 4% SDS). This mixture was left at room temperature for 30 minutes. 10mls of TE-saturated phenol (phenol saturated with 10mM Tris, 1mM EDTA) were then added. The tube was centrifuged at 1000g for 20 minutes.

In the class I cabinet, the upper layer was removed into a clean tube. 2.5mls 7.5M ammonium acetate and 15mls cold isopropanol were added. The sample was mixed and stored overnight at -20°C.

The following day, the tube was centrifuged at 1000g for 30 minutes. The alcohol was discarded, and the tube inverted to allow it to drain. 2.5mls de-ionised distilled

water containing 1·25mg Proteinase K was added. The tube was incubated at 48°C overnight.

An equal volume of TE-saturated phenol was added. This was then mixed thoroughly, and the sample centrifuged at 1000g for 5 minutes. The upper layer was removed into a clean tube. An equal volume of chloroform:iso-amyl alcohol (24:1) was added, mixed thoroughly and the tube was then centrifuged at 1000g for 5 minutes. The upper layer was removed into a clean tube. 0·125mls 7·5M ammonium acetate and twice the volume of cold absolute ethanol were added. This was mixed thoroughly and stored for 24 hours at -20°C.

The tube was centrifuged at 1000g for 20 minutes, the alcohol removed and the tube inverted to drain. The DNA was re-suspended in 250µl of distilled water.

In order to calculate the DNA concentration of this solution, 10µl of it was taken and diluted further to a volume of 1000µl. The optical density (OD) of this solution at 260nm was measured with a spectrophotometer. An OD=1 is equivalent to a DNA concentration of 50ng/µl, therefore the DNA concentration of the original solution was calculated as follows :

$$\text{DNA concentration (ng/}\mu\text{l)} = \text{OD}_{260\text{nm}} \times 50 \times \frac{1000}{10}$$

An aliquot was then made to give a final concentration of 50ng/µl.

The quality of the DNA was not specifically tested.

4.6 DNA Extraction From Paraffin Blocks

The method described below for extraction of DNA from archival wax-embedded tissue was modified from a method that has been described previously (Levi et al,

1991). This is a relatively rapid method of DNA extraction that avoids the use of xylene and ethanol, which remove paraffin from the sections. It can provide a good yield of DNA from a limited amount of tissue.

A 4µm section was cut from the paraffin block using a microtome. This was then placed onto a microscope slide and dried at 60°C for 45 minutes. The slide was stained with haematoxylin and eosin (see below).

The slide was then studied by a pathologist (Dr. K. Kurian) and the areas of tumour (if present) and normal tissue in the section were identified and marked.

Between two and four (depending upon the area of tissue) 10µm sections were cut from the paraffin block. If present, normal tissue was cut out for use. If no normal tissue was present, then tumour tissue was used. The sections were then placed in 400µl X1 lysis buffer (10mM Tris, 50mM KCl, 0.45% Tween20, 2.5mM MgCl₂) containing 0.5mg Proteinase K. This was incubated at 55°C for 6-8 hours. It was then boiled for 20 minutes to denature the DNA and destroy the Proteinase K. 1µl 0.5M EDTA was added. The solution was divided into 50-100µl aliquots. One of these was stored at 4°C for immediate use and the remainder were stored at -20°C. DNA concentrations were not measured.

4.7 Haematoxylin and Eosin Staining

The xylene, haematoxylin, eosin and different concentrations of ethanol used during each part of the following process were in separate glass staining troughs.

Slides onto which paraffin sections had been dried were placed into a rack. The rack was placed in xylene for 5 minutes to de-wax the sections. The slides were then drained, placed in absolute ethanol for 1 minute followed by 70% ethanol for 1

minute and then 60% ethanol for 1 minute. The slides were rinsed in tap water for 2 minutes. The surface of the haematoxylin was skimmed with a paper towel in order to remove oxidised particles. The slides were then placed into the haematoxylin for 5 minutes. The residual haematoxylin was rinsed off with water and the slides were dipped into 1% acid alcohol (1ml hydrochloric acid in 100ml absolute ethanol) for 5 seconds. They were rinsed with water for 1 minute, then placed in eosin for 30 seconds and rinsed with water for 1 minute. The slides were placed in 60% ethanol for 1 minute, followed by 70% ethanol for 1 minute and then absolute ethanol for 1 minute.

If the staining appeared to be too dark, then the slides were placed in acid alcohol for 5 seconds. If the staining appeared too light, then the haematoxylin and eosin staining was repeated.

The rack containing the slides was placed in xylene ready for mounting. DPX synthetic resin mountant (a mixture of distyrene, a plasticizer and xylene) was applied to a coverslip, the slide drained and then lowered onto the coverslip. Any air bubbles between the slide and coverslip were then pressed out.

4.8 Polymerase Chain Reaction (PCR)

Reaction mixtures were set up within a Class I microbiological safety cabinet in order to minimise the risk of contamination. The constituents of the reaction mixture depended on the primers used for each gene under study. These details are therefore given later in the chapter in sections 4.15 - 4.19.

All reactions were performed in 0.5 ml Eppendorf tubes and the mixture in each case was overlaid with an equal volume (25 or 50 μ l) paraffin oil. With all sets of reactions, a single tube containing no DNA was included in order to check for contamination of the reaction constituents.

In all cases, a 'hot-start' protocol was used. The principle of this is that the template DNA and the primers are mixed together and held at a temperature above the threshold of non-specific binding of primer to template, prior to addition of the Taq DNA polymerase. Just before the cycling part of the reaction, polymerase is added to allow the reaction to take place at higher temperature. The primers therefore do not have the chance to anneal non-specifically to the template. Consequently, the amplified DNA bands tend to be cleaner.

Using an OmniGene thermal cycler (Hybaid, UK) the initial denaturation step of the PCR was performed at 94°C for 3 minutes. The tubes were then cooled down and held at a temperature of 80°C. The Taq DNA polymerase was then inserted into each tube (below the layer of oil) at this stage. The cycling process was then commenced.

In order to optimise the conditions for each PCR, DNA extracted from blood was used. The concentration of certain constituents of the reaction were varied. The concentration of MgCl₂ was varied between 1mM and 2mM. The amount of each primer used was varied between 10pmol and 100pmol. The concentration of deoxynucleoside triphosphates was varied between 100 μ M and 200 μ M. The annealing temperature for each reaction was initially based upon published data where primers had been used in other studies, or the predicted annealing temperature (Primer Designer v1.1 ©1990 Educational Software) minus 5°C where primers were

designed *de novo*. The annealing temperature was then lowered in 1°C stages if the PCR was unsuccessful, or raised in 1°C stages if there were too many non-specific products.

It was found that some of the DNA samples extracted from paraffin sections did not amplify well. In these cases, the PCR was repeated up to five times if necessary. The volume of DNA solution added was increased up to 5µl once the other conditions had been optimised using samples containing higher concentrations of DNA. It was also found that lowering the annealing temperature by up to 5°C was helpful in some cases.

4.9 Agarose Gel Electrophoresis

TBE buffer was prepared initially as a 10x stock, containing 0.89M Tris base, 0.89M Boric acid and 20mM EDTA (pH8.0). 100mls x1 TBE buffer was added to 3g of agarose powder in a conical flask. The mixture was heated in a microwave oven for a total of 90 seconds, with mixing after 30 and 60 seconds. The molten agarose was then allowed to cool to 60°C. 5µl ethidium bromide (10µg/µl) was added and the agarose poured into a slab gel mould. Combs were inserted and the gel was allowed to set.

Once the gel had set, the combs were removed and the gel placed in a tank with x1 TBE buffer.

All PCR products were run on an agarose gel to confirm that the PCR had worked successfully. 3µl Xylene Cyanol/Bromophenol Blue loading buffer was mixed with 10µl of each sample and loaded onto the gel. Electrophoresis was at 125volts for

approximately 30 minutes. The gel was then visualised under ultraviolet light and if the DNA had not run far enough down the gel, electrophoresis was continued.

4.10 Heteroduplex Analysis

A set of two glass plates was cleaned thoroughly with cream cleanser. The plates were then rinsed thoroughly with tap water followed by 95% ethanol. They were then dried with a paper towel. Prior to their first use, the shorter of the two plates was marked 'BIND' with a diamond-tipped pen and the longer plate was marked 'SLICK'.

In a Class I microbiological safety cabinet, silane solution (1ml 95% ethanol, 5 μ l glacial acetic acid, 3 μ l γ -Methacryloxypropyltrimethoxysilane) was applied thoroughly to the 'BIND' plate. This plate was then wiped clean with 95% ethanol four times using a fresh paper towel each time. With approximately every tenth use of the plates, 3mls of gel slick were applied to the 'SLICK' plate after having changed gloves. This was left for between five and ten minutes. The excess gel slick was then wiped off with distilled water, and the plate dried with a paper towel. The plates were assembled with 0.4mm spacers.

To make 50mls of non-denaturing 1x Sequagel[®] MD gel, 25mls Sequagel[®] MD (2x) was mixed with 7.5g urea, 3ml x10 TBE and 17.5mls ultrapure deionised distilled water.

When ready for use, 400 μ l of 10% Ammonium Persulfate (APS) (not more than one week old) and 40 μ l of Tetramethylethylenediamine (TEMED) were added. The gel was then poured in between the plates using a 50ml syringe. A square tooth comb

was placed in one end of the gel and secured with clamps. The gel was then allowed to set for approximately one hour.

The plates were assembled vertically in S2 Sequencing gel apparatus (Life Technologies[™]) and a total of 1 litre of 0.6x TBE poured into the upper and lower chambers. The comb was removed and the gel wells were flushed out with 0.6x TBE using a syringe.

The PCR products were denatured for 3 minutes at 95°C and then allowed to cool to 37°C over 30 minutes using a program on an OmniGene thermal cycler (Hybaid, UK).

2 - 5µl of bromophenol blue loading buffer were added to 4-10µl of each sample and these were then loaded onto the gel. A sample known to produce heteroduplexes was also loaded as a control. Electrophoresis was for approximately 16 hours at 500 volts.

4.11 Silver Staining

The gel was placed into a litre of 10% ethanol (100mls absolute ethanol in 900mls ultrapure deionised distilled water) and left on a rocking platform for 10 minutes.

The ethanol was then discarded and a litre 1% nitric acid (10mls concentrated nitric acid in 1 litre ultrapure deionised distilled water) was added. This was returned to the rocking platform for 10 minutes. The plate was rinsed in ultrapure deionised distilled water for 5 minutes and then 1 litre silver nitrate (2.04g in 1 litre ultrapure deionised distilled water) was added. This was then left on the rocking platform for 20 minutes.

The silver nitrate was returned to its bottle and the gel was rinsed quickly (for 10 seconds) in distilled water.

The plate was then rinsed in 1 litre of pre-cooled developer (160g sodium carbonate decahydrate, 1ml formaldehyde, 2 litres ultrapure deionised distilled water, cooled to 4°C). The developer was poured off when a precipitate developed and then replaced with another litre of fresh developer. The gel was returned to the rocking platform until bands appeared. The reaction was then stopped by adding 1 litre 0.1M citric acid (19.2g in 1 litre ultrapure deionised distilled water). This was left for 10 minutes and the plate was then rinsed for 10 minutes in distilled water. The plate was then allowed to dry. It was placed on a flatbed scanner and an image transferred onto a personal computer.

4.12 Cycle Sequencing

Sequencing was performed using Thermo Sequenase[™] radiolabelled terminator cycle sequencing kits (Amersham Life Science).

Sequencing of PCR products was usually performed in batches of six. 7µl of each PCR product to be sequenced was taken and to this, 1µl Exonuclease I and 1µl Shrimp Alkaline Phosphatase were added. The tubes (labelled 1 to 6) were heated to 37°C for 15 minutes, then to 80°C for 15 minutes.

24 0.5 ml Eppendorf tubes were taken and labelled G1 to G6, A1 to A6, T1 to T6 and C1 to C6.

These tubes were placed on ice. 12µl of dGTP nucleotide master mix was pipetted into each of the tubes G6, A6, T6 and C6. 3µl of [α -³³P]ddGTP was pipetted into tube G6, 3µl of [α -³³P]ddATP into tube A6, 3µl of [α -³³P]ddTTP into tube T6 and 3µl of [α -³³P]ddCTP into tube C6. 2.5µl of the mixture from tube G6 was transferred

into each of the tubes G1 to G5. The same process was performed with the tubes A6, T6 and C6.

The 6 cleaned PCR products were taken and to each tube, 2µl Reaction Buffer, 1µl Primer, 10µl DDW and finally 2µl Thermo Sequenase Polymerase were added. 4.5µl of this reaction mixture from PCR tube 1 was transferred into each termination tube (G1,A1,T1 and C1). The same process was performed for each of the remaining PCR tubes 2 to 6. The contents of each of the 24 tubes were mixed well and overlaid with 20µl of oil.

The 24 tubes were then put onto an OmniGene thermal cycler (Hybaid, UK) and cycled 38 times at 94°C for 45 seconds, T_a °C for 45 seconds and at 72°C 45 seconds (where T_a was the annealing temperature used in the original PCR).

4µl STOP solution (95% formamide, 10mM EDTA pH8.0, 0.01% bromophenol blue, 0.01% xylene cyanol) was added to each of the termination reactions, they were mixed thoroughly and then centrifuged briefly to separate from the oil.

Prior to use, the samples were heated to 95°C for 5 minutes and then cooled rapidly on ice.

For each of the samples a 'long run' and a 'short run' was performed.

Electrophoresis was on a denaturing gel (see below) at 70 to 80 watts for 2-3 hours ('long run') and 1-1½ hours ('short run').

While still warm, the gel was transferred onto filter paper and then placed in a gel drier. Within a darkroom, the dried gel was placed against the emulsion surface of an autoradiography film, and the gel and film placed together into a light-proof cassette. This was left overnight and the film was developed the following day.

4.13 Denaturing Gel Electrophoresis

A 6% acrylamide stock solution was prepared by mixing 240g urea with 75mls 40% acrylamide, 190ml deionised distilled water and 50ml 10x TBE for about 30-60 minutes using a magnetic stirrer.

If the gel was to be silver-stained, then the plates were prepared as described in the previous section 'Heteroduplex Analysis' and assembled with 0.4mm spacers. For a sequencing gel, a short plate free from bind solution was used.

For each gel, 75mls of the stock solution was taken and 500 μ l of 10% APS and 50 μ l of TEMED were added.

The gel was poured in between the gel plates and a sharks-tooth comb inserted. The comb was secured with clamps and the gel was allowed to set for 40-60 minutes.

The plates were assembled vertically in S2 Sequencing gel apparatus (Life Technologies[™]) and 0.5x TBE poured into the top and bottom chambers (1 litre in total was used). The comb was inverted and inserted until the teeth penetrated approximately 2mm into the gel. The wells were flushed out with 0.5x TBE.

The gel was pre-run at 70watts to warm it for about 60 minutes.

While the gel was warming, the samples were prepared. 2.5 μ l of STOP solution was put into the required number of tubes. 5 μ l of each sample was added to a tube. These were heated to 95°C for 5 minutes and then cooled rapidly on ice.

The wells were flushed out again and 5 μ l of each sample loaded into the wells.

Electrophoresis was at 70watts until the xylene cyanol reached the bottom of the gel (except when used for cycle sequencing – see above).

4.14 Protein Truncation Testing (PTT)

Using primers selected from those used for amplification of the BRCA2 gene, overlapping PCR products of 0.8-1.4 kb in size were generated, covering BRCA2 exons 10, 11 and 27. PTT forward primers described by Lancaster et al (1996) were then used for nested PCR.

The TnT[®] Quick Coupled Transcription/Translation System (Promega) was used for the *in vitro* generation of protein products from PCR amplified DNA. Reaction mixes containing 10 μ l TnT[®] Quick Master Mix (Promega), 0.25 μ l 1mM Methionine, 0.5 μ l Transcend[™] Biotin-Lysyl-tRNA and 2 μ l secondary PCR product were incubated at 30°C for 90 minutes.

A Mini-Protean[®] II Electrophoresis Cell (Biorad) was used for electrophoresis of the samples. This allows two gels to be run simultaneously. Two sets of glass plates, spacers and combs were assembled within a casting stand. A 12% polyacrylamide gel mixture was made by mixing 4.1mls deionised distilled water, 8mls 30% acrylamide, 7.5mls 1M Tris pH 7.5, 200 μ l 10% SDS, 200 μ l 10% APS and 8 μ l TEMED. The gel mixture was then poured between both sets of glass plates and left to polymerise for 45-60 minutes. The combs were removed and the wells rinsed thoroughly with distilled water. The electrophoresis cell containing the two gels was then assembled. A 10X stock of SDS-PAGE running buffer was made by mixing 30g Tris base, 144g glycine, 100mls 10% SDS and deionised distilled water to a volume of one litre. 300mls 1X SDS-PAGE buffer was then prepared and used to fill the chambers of the electrophoresis cell.

950 μ l loading dye was mixed with 50 μ l β -Mercaptoethanol. 20 μ l of this mixture was then added to 12.5 μ l of each sample. 20 μ l loading dye was also added to 0.5 μ l ladder

(Biotinylated SDS-PAGE Low Range Standard, Biorad). These samples were denatured at 95°C for 3 minutes and then loaded onto the gels. Electrophoresis was performed at 35mA for about 3 hours, until the dye had reached the bottom of the gel.

The proteins were electroblotted onto nitrocellulose membranes using a Trans-Blot[®] Electrophoretic Transfer Cell (Biorad). A 20X stock of transfer buffer was made by mixing 29g glycine with 58g Tris, and making up to 500mls with deionised distilled water. Just prior to use, 2l of buffer was made by mixing 100mls of the 20X stock solution with 400mls methanol and 1500 mls deionised distilled water. Nitrocellulose membrane and filter paper were cut to the same size as the two gels. The membrane, filter paper and fibre pads were soaked in the transfer buffer for 15 minutes. A fibre pad was placed onto the Trans-Blot[®] cassette. A piece of filter paper was then placed onto this, followed by the gel, then the membrane and then a second piece of filter paper. Any air bubbles were removed and the second fibre pad placed on top. The cassette was closed and placed into the tank, which was then filled with buffer. The blotting was performed overnight at 25volts.

The proteins were then visualised using the Transcend[™] Colorimetric Translation Detection System (Promega). After the blotting was completed, each wet membrane was rinsed in tris-buffered saline (TBS), taking care to keep the side of the membrane containing the proteins facing upwards. 15mls of TBS + 0.5% Tween[®]20 were added to the membrane left for 60 minutes at room temperature. 6µl Streptavidin alkaline phosphatase (Streptavidin-AP) was diluted with 15mls fresh TBS + 0.5% Tween[®]20. The previous solution of TBS + 0.5% Tween[®]20 was

poured off and the Streptavidin-AP solution was added to the membrane. This was placed on a rocking platform for a further 60 minutes. The Streptavidin-AP solution was poured off and the membrane washed twice for one minute with 30mls TBS + 0.5% Tween[®]20, followed by two washes for one minute with 30mls TBS. The membrane was incubated in about 5mls Western Blue[®] Stabilized Substrate for Alkaline Phosphatase until the bands had reached the required intensity. The reaction was stopped by washing the membrane in two baths of deionised distilled water for 2 minutes. The membrane was dried by exposure to air and stored protected from light. Detailed instructions for the use of the TnT[®] Quick Coupled Transcription/Translation System (Promega), the Mini-Protean[®] II Electrophoresis Cell (Biorad), the Trans-Blot[®] Electrophoretic Transfer Cell (Biorad) and the Transcend[™] Colorimetric Translation Detection System (Promega) can be found in their corresponding instruction manuals (see references).

The details of methods specific to each of the genes studied are now described below.

4.15 Androgen Receptor Gene

Using the published sequence (see references), the following primers were designed (Primer Designer v1.1 ©1990 Educational Software) and manufactured by Genosys Biotechnologies (Europe) Ltd. :

ARG-F 5'-TGCGCGAAGTGATCCAGAACC-3'

ARG-R 5'-CTCATCCAGGACCAGGTAGCC-3'

These generate PCR fragments containing the CAG repeat sequence.

PCR reactions were performed in 50µl aliquots, each containing 31µl de-ionised distilled water, 1x PCR reaction buffer (5µl of 10x concentrate used, containing 200mM Tris-HCl pH8.4 and 500mM KCl, Life Technologies™), 2mM (2µl) MgCl₂, 5µl Dimethyl Sulfoxide, 200µM (1 µl) deoxynucleoside triphosphates, 20 pmol of each primer (supplied primers diluted to 20pmol/µl and 1µl used), 1 unit of Taq polymerase (supplied as 5 units/µl, diluted 1:10 and 2µl used, Life Technologies™) and approximately 100ng (2µl) DNA. The amplification was performed using an OmniGene thermal cycler (Hybaid, UK) under the following conditions: initial denaturation at 94°C for 3 minutes; amplification for 38 cycles, with denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds and extension at 72°C for 45 seconds; final extension at 72°C for 10 minutes.

The products were denatured and then run on 6% polyacrylamide gels with a 10bp DNA ladder (range 10bp – 330bp, Life Technologies™). The products were then ranked in order of length. Cycle sequencing of products was unsuccessful. Three representative products were therefore sent for automated sequencing (DNASHEF, Dept. of Haematology, Royal Infirmary of Edinburgh) to confirm the number of CAG repeats, and these were used as size standards. The products were then re-run, with those thought to be of equal length adjacent to each other in order to check the accuracy of the original estimation of length. A second re-run was then performed to confirm the accuracy of the results. The number of CAG repeats in each sample could then be calculated.

The distribution of alleles of the androgen receptor gene, comparing the male breast cancer patients with 79 male controls, was analysed using the Mann-Whitney test.

4.16 BRCA1 & BRCA2

The entire coding region and splicing sites of BRCA2 was amplified by polymerase chain reaction using 62 pairs of primers (designed by Dr. R. Wooster; sequences provided by Dr. N. Spurr, personal communication). These primers are listed in Appendix 7.2 and were manufactured by Life TechnologiesTM. A region of BRCA1 was also amplified (using primers shown in Appendix 7.3) to screen for the 2800delAA mutation that is thought to be a founder mutation in Scotland (Renwick et al, 1997; Liede et al, 2000). Reactions were performed in 25µl aliquots, each containing 13.5µl de-ionised distilled water, 1x PCR reaction buffer (2.5µl of 10x concentrate used, containing 200mM Tris-HCl pH8.4 and 500mM KCl, Life TechnologiesTM), 2mM (1µl) MgCl₂, 5µl Dimethyl Sulfoxide, 200µM (0.5µl) deoxynucleoside triphosphates, 10 pmol of each primer (supplied primers diluted to 10pmol/µl and 1µl used), 0.5 units of Taq polymerase (supplied as 5 units/µl, diluted 1:20 and 2µl used, Life TechnologiesTM) and approximately 50ng (1µl) DNA. The amplification was performed using an OmniGene thermal cycler (Hybaid, UK) under the following conditions: initial denaturation at 94°C for 3 minutes; amplification for 35 cycles, with denaturation at 94°C for 45 seconds, annealing at 45-60°C (depending upon the primer used) for 45 seconds and extension at 72°C for 45 seconds; final extension at 72°C for 10 minutes. The annealing temperature for each pair of primers is shown in Appendix 7.2. Where two temperatures are given then a touchdown PCR was found to work better, with five amplification cycles at the higher temperature, followed by 35 cycles at the lower temperature.

PCR products underwent Heteroduplex analysis and were visualised by staining with silver nitrate. BRCA2 exons 10, 11 and 27 were also studied by PTT.

PCR products showing variant bands on heteroduplex analysis or PTT were re-amplified from the genomic DNA and then sequenced. The sequences produced were compared to the published BRCA2 sequence (details of the exonic and flanking intronic sequences can be found on the Breast Cancer Information Core).

When mutations and polymorphisms were detected, new primers were designed (Primer Designer v1.1 ©1990 Educational Software) in order to produce shorter PCR products covering those areas. This was done in order to maximise the number of archival samples containing poor quality DNA that could be screened for mutations already detected in those samples containing better quality DNA. These primers are listed in Appendix 7.3.

In designing these primers, a number of factors needed to be taken into account. The computer software used was able to check that the primers designed would not hybridise to each other and that they should also hybridise to the genomic DNA under study at similar temperatures. It was also important to design primers that did not contain long runs of a single nucleotide and whose base composition was 50-60% GC-rich. It is sufficient for primers to be about 20 nucleotides in length, because the chances of a perfect match elsewhere in the genome is then extremely low.

Shortening the length of the PCR product produced will mean a greater chance that the PCR will be successful when the DNA used is of poor quality. However, it has been shown that the difference in migration between heteroduplexes and homoduplexes is reduced when the PCR product length is less than 150bp (Ganguly et al, 2002) which would reduce the sensitivity of heteroduplex analysis. It is also



important to design primers so that the mutation or polymorphism being screened for is not too close to one end of the PCR product. If this was the case, then the sequence change may not alter the conformation of the DNA sufficiently to allow detection by heteroduplex analysis (Rossetti et al, 1995). It was obviously important to ensure that heteroduplex analysis could detect sequence changes in PCR products produced using the new primers. The DNA samples in which mutations had been detected with the original primers were therefore taken, amplified using the new primers, and used as positive controls for the heteroduplex analysis.

4.17 CYP17

The primers originally described produce a PCR product of 459bp in length (Carey et al, 1994). This would clearly restrict the numbers of successful PCR's using DNA extracted from archival tissue.

The following primers were designed for PCR (Primer Designer v1.1 ©1990 Educational Software) and manufactured by Life Technologies™ :

CYP17S-F, 5'-CAAAAGTCAAGGTGAAGATCAG-3'

CYP17S-R, 5'-TAGGGTAAGCAGCAAGAGAG-3'

These generate PCR fragments 150bp in length, including the polymorphic site. PCR reactions were performed in 50µl aliquots, each containing 31.5µl de-ionised distilled water, 1x PCR reaction buffer (5µl of 10x concentrate used, containing 200mM Tris-HCl pH8.4 and 500mM KCl, Life Technologies™), 2mM (2µl) MgCl₂, 100µM (0.5µl) deoxynucleotide triphosphates, 100 pmol of each primer (supplied primers diluted to 100pmol/µl and 1µl used), 1 unit of Taq polymerase (supplied as 5 units/µl, diluted 1:10 and 2µl used, Life Technologies™) and approximately 100ng

(2µl) DNA. The amplification was performed using an OmniGene thermal cycler (Hybaid, UK) under the following conditions : initial denaturation at 94°C for 3 minutes; amplification for 38 cycles, with denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds and extension at 72°C for 45 seconds; final extension at 72°C for 10 minutes.

10µl of each PCR product was run on an agarose gel to confirm that the PCR had been successful. To the remaining 40µl of each PCR product were added 0.2µl restriction enzyme MspA1 (Promega) and 4.5µl 10X Multi-Core™ Buffer (1x = 25mM Tris-Acetate, pH 7.5 at 37°C, 100mM potassium acetate, 10mM magnesium acetate, 1mM DL-Dithiothreitol). The PCR products were digested for 90 minutes at 37°C. The products were separated by electrophoresis on a 4% agarose gel, staining with ethidium bromide and identified from photographs of the gel under ultraviolet illumination.

The association between the C allele of the CYP17 gene and breast cancer in the male and female subjects and controls was analysed using χ^2 tests.

4.18 CYP19

Primers as previously described (Polymeropoulos et al, 1991) were manufactured by Genosys Biotechnologies (Europe) Ltd. and used for PCR :

CYP19-F : 5'-GCAGGTACTTAGTTAGCTAC-3'

CYP19-R : 5'-TTACAGTGAGCCAAGGTCGT-3'

These generate PCR fragments that include the polymorphic site. PCR reactions were performed in 50µl aliquots, each containing 36µl de-ionised distilled water, 1X PCR reaction buffer (5µl of 10x concentrate used, containing 200mM Tris-HCl

pH8.4 and 500mM KCl, Life Technologies[™]), 2mM (2µl) MgCl₂, 200µM (1µl) deoxynucleoside triphosphates, 40 pmol of each primer (supplied primers diluted to 40pmol/µl and 1µl used), 1 unit of Taq polymerase (supplied as 5 units/µl, diluted 1:10 and 2µl used, Life Technologies[™]) and approximately 100ng (2µl) DNA. The amplification was performed using an OmniGene thermal cycler (Hybaid, UK) under the following conditions : initial denaturation at 94°C for 3 minutes; amplification for 38 cycles, with denaturation at 94°C for 45 seconds, annealing at 53°C for 45 seconds and extension at 72°C for 45 seconds; final extension at 72°C for 10 minutes.

The products were denatured and then run on 6% polyacrylamide gels with a 10bp DNA ladder (range 10bp – 330bp, Life Technologies[™]). The number of TTTA repeats in homozygous products were measured by cycle sequencing and these were used as size standards.

The distribution of alleles of the CYP19 gene, comparing male breast cancer patients with controls, was analysed using the Mann-Whitney test. Odds ratios with 95% confidence intervals were calculated to show the risk of developing male breast cancer associated with each allele.

4.19 Oestrogen Receptor Gene

The oestrogen receptor gene intron 1 sequence was provided by Dr. Alison Dunning (personal communication). The following primers were designed (Primer Designer v1.1 ©1990 Educational Software) for PCR amplification of the relevant fragment (128bp) of intron 1 containing the polymorphic site :

ERG1-F : 5'-CATGTTCTGTGTTGTCCATC-3'

ERG1-R : 5'-GAACCATTAGAGACCAATGC-3'

Primers as previously described (Andersen et al, 1994) were used for PCR amplification of the relevant fragment of exon 2. Both pairs of primers were manufactured by Genosys Biotechnologies (Europe) Ltd.

PCR reactions were performed in 50µl aliquots, each containing 36µl (intron 1) or 37µl (exon 2) de-ionised distilled water, 1X PCR reaction buffer (5µl of 10x concentrate used, containing 200mM Tris-HCl pH8.4 and 500mM KCl, Life Technologies™), 2mM (2µl) MgCl₂ (intron 1) or 1mM (1µl) MgCl₂ (exon 2), 200µM (2µl) deoxynucleoside triphosphates, 20 pmol of each primer supplied primers diluted to 20pmol/µl and 1µl used), 1 unit of Taq polymerase (supplied as 5 units/µl, diluted 1:10 and 2µl used, Life Technologies™) and approximately 100ng (2µl) DNA. The amplification was performed using an OmniGene thermal cycler (Hybaid, UK) under the following conditions : initial denaturation at 94°C for 3 minutes; amplification for 38 cycles, with denaturation at 94°C for 45 seconds, annealing at 54°C (intron 1) or 56°C (exon 2) for 45 seconds and extension at 72°C for 45 seconds; final extension at 72°C for 10 minutes.

The intron 1 products were digested overnight by a restriction enzyme, PvuII (New England Biolabs®) at 37°C and the exon 2 products by BstUI (New England

Biolabs®) at 60°C. The products were then separated on 4% agarose gels, stained with ethidium bromide and visualised under ultraviolet light.

The distributions of alleles of the oestrogen receptor gene, comparing male breast cancer patients with controls, were analysed using χ^2 tests. Odds ratios with 95% confidence intervals were calculated to show the risk of developing male breast cancer associated with each polymorphism.

Chapter 5 - Results

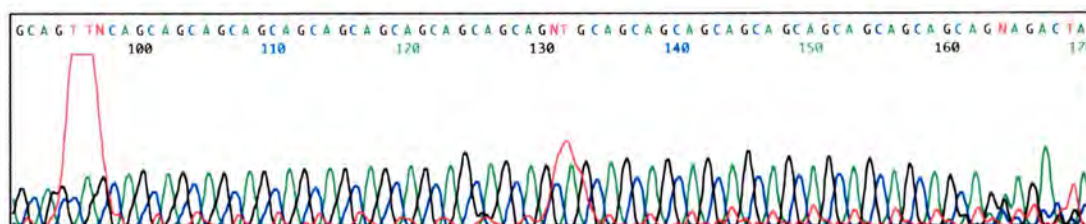
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5.1 Androgen Receptor Gene

The lengths of the PCR products obtained varied between 224 bp and 272 bp (corresponding to 14 CAG repeats and 30 CAG repeats respectively). PCR was unsuccessful with DNA extracted from eight of the archival wax-embedded tissue sections. An example of the automated sequencing of a CAG repeat sequence is shown in Figure 5·1. The distribution of alleles among male breast cancer patients and controls is shown in Figure 5·2. The median number of CAG repeats in both groups was 23. There were no statistically significant differences between the two groups (Mann-Whitney test, $p=0·916$).

Figure 5.1

Automated sequencing of a PCR product containing 25 CAG repeats



Three patients showed evidence of two different alleles indicating the presence of two X chromosomes (Figure 5.3). One of these (MBC17) was recorded on the Edinburgh Cytogenetics Register with a diagnosis of Klinefelter's Syndrome. The other two patients (MBC42 & MBC54) were deceased, but there was no record of clinical suspicion of Klinefelter's syndrome in their hospital casenotes. Neither fathered any children. The data were re-analysed following exclusion of these three cases. The median number of CAG repeats for the remaining 53 male breast cancer

patients was also 23. There were still no statistically significant difference between cases and controls ($p=0.765$).

Figure 5.2

Distribution of alleles of the androgen receptor gene among male breast cancer patients and controls

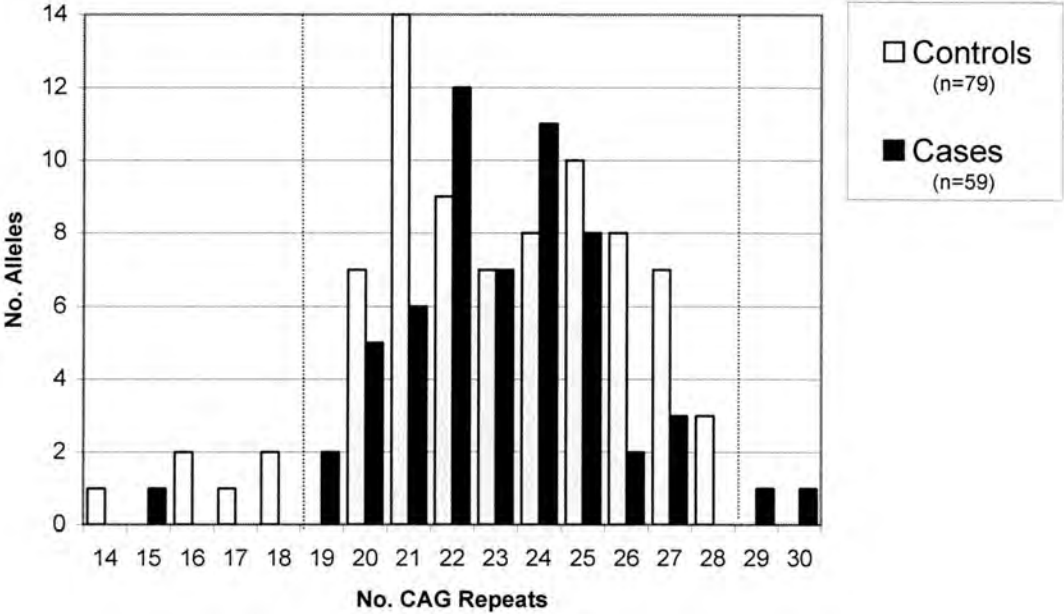
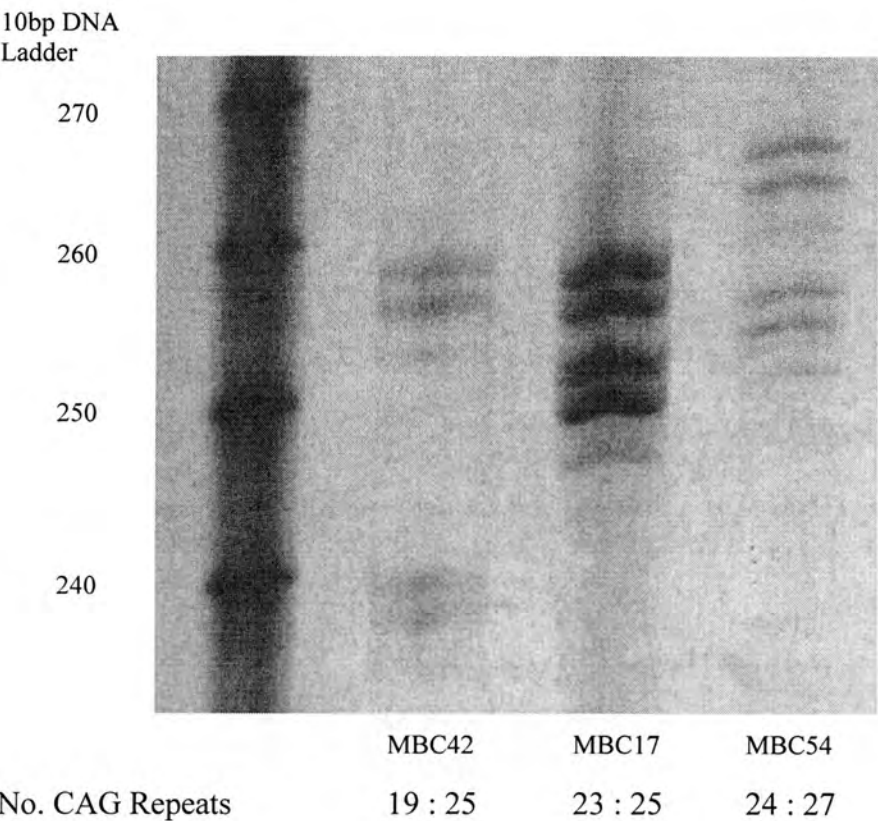


Figure 5.3

Three of the male breast cancer cases, each showing two different alleles of the androgen receptor gene



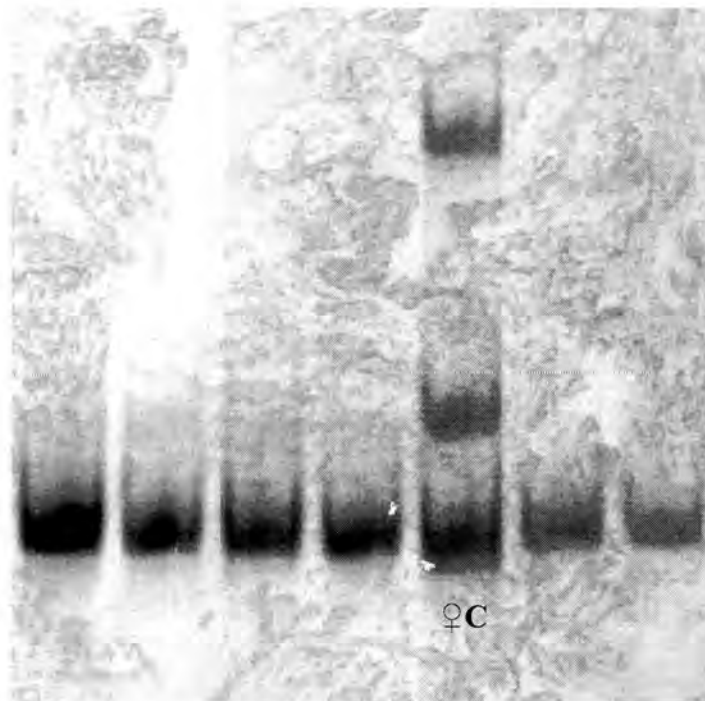
5.2 BRCA1 & BRCA2

52 of the 64 male breast cancer cases were screened successfully for the BRCA1 2800 delAA mutation. None produced variant bands on heteroduplex analysis (HA) and therefore no BRCA1 2800 delAA mutations were detected.

HA of a number of PCR products generated to screen for this mutation are shown in Figure 5.4. DNA from a female known to carry the mutation was provided by Dr. B. Cohen (School of Biomedical Sciences, University of St. Andrews). The PCR product generated from this DNA sample clearly produced variant bands as shown and was therefore a useful positive control.

Figure 5.4

Heteroduplex analysis to screen for BRCA1 2800delAA mutation



♀C = Female 'control' known to carry BRCA1 2800 delAA mutation

For the screening of the BRCA2 gene, more than 85% of polymerase chain reactions were ultimately successful. The DNA derived from certain archival samples was clearly not of good quality. The results of BRCA2 mutation analysis are shown in Table 5-1. Germline BRCA2 mutations were identified in 12 of the 64 (19%) male breast cancer cases. None of these mutations were detected in the control population. Figure 5-5 demonstrates the detection of a number of these mutations on HA and Figure 5-6 shows one of the mutations detected on PTT. The sequencing of DNA demonstrating each of these mutations are shown in Figures 5-7 - 5-13. These sequences are read 5'→3' from the bottom of the figure upwards. Exonic sequences are given in uppercase, intronic sequences in lowercase.

A number of polymorphisms in BRCA2 were also detected. The frequency of these among male breast cancer cases and controls is shown in Table 5-2.

38 / 61 (62%) of the male breast cancer patients carry the 203 G→A exon 2 polymorphism. 52 / 116 of the controls (45%) carry the polymorphism. The difference between cases and controls is statistically significant ($p=0.0272$, $\chi^2=4.88$, 1 d.f.). The odds ratio for the risk of a male with the exon 2 polymorphism developing breast cancer is calculated as 2.033 (95% confidence interval 1.079 – 3.834). All three male breast cancer cases carrying the 7830 del16 exon 16 mutation also carry the exon 2 polymorphism.

Family pedigrees of the 12 males found to carry BRCA2 mutations are shown in Figures 5-14 to 5-25. Six of these cases (50%) had a history of breast cancer in a first- or second-degree relative.

Table 5.1

Mutations detected in the BRCA2 gene

Mutation	Location	Putative Coding Effect	No. Cases	Age at Diagnosis
1418 insTTAG	Exon 10	Frameshift	2	55, 71
5910 C→G	Exon 11	Tyr → STOP	1	66
7165+2 delT	Intron 12	Splicing	2	32, 81
7830 del16	Exon 16	Frameshift	3	73, 75, 79
8560-1 G→C	Intron 18	Splicing	1	39
9522 C→G	Exon 25	Tyr → STOP	1	62
9877-67 delTTAC	Intron 26	Splicing	2	56, 66

Table 5·2

Polymorphisms detected in the BRCA2 gene among cases and controls

Polymorphism	Location	Freq. Cases	Freq. Controls	p (χ^2 , 1 d.f.)
203 G→A	Exon 2	38 / 61	52 / 116	0·0272
3199 A→G	Exon 11	2 / 60	8 / 58	0·0609
4035 T→C	Exon 11	12 / 61	21 / 80	
5972 C→T	Exon 11	7 / 61	5 / 34	
7470 A→G	Exon 14	11 / 55	Not done *	
8034-14 T→C	Intron 16	5 / 64	5 / 43	
9485-16 T→C	Intron 24	1 / 51	2 / 42	

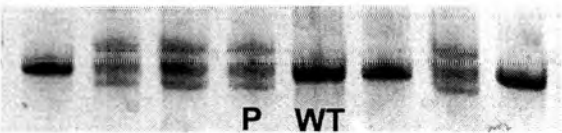
* There was insufficient time at the end of the period of study to determine the frequency of this polymorphism in controls

Missense sequence variants were found on sequencing 17 of the PCR products that had produced variant bands on HA. However, none of these were found a second time after repetition of the PCR's. They were all in DNA extracted from archival tissue. They were therefore presumed to be due to errors within the PCR process rather than the HA.

Figure 5.5

Examples of polymorphisms and mutations detected by heteroduplex analysis

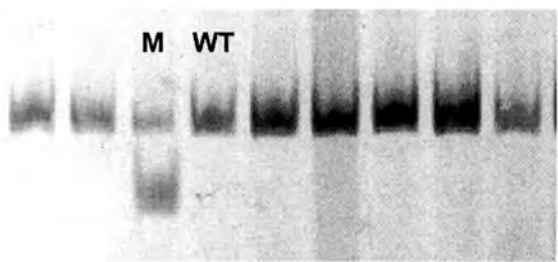
203 G→A Polymorphism (Exon 2)



1418 insTTAG Mutation (Exon 10)

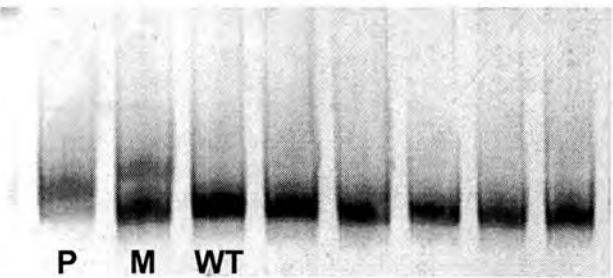


7830 del16 Mutation (Exon 16)



9485-16 T→C Polymorphism (Intron 24)

9522 C→G Mutation (Exon 25)



P = Polymorphism

M = Mutation

WT = Wild-type

Figure 5-6

A truncating mutation in exon 11 of BRCA2 demonstrated by protein truncation test

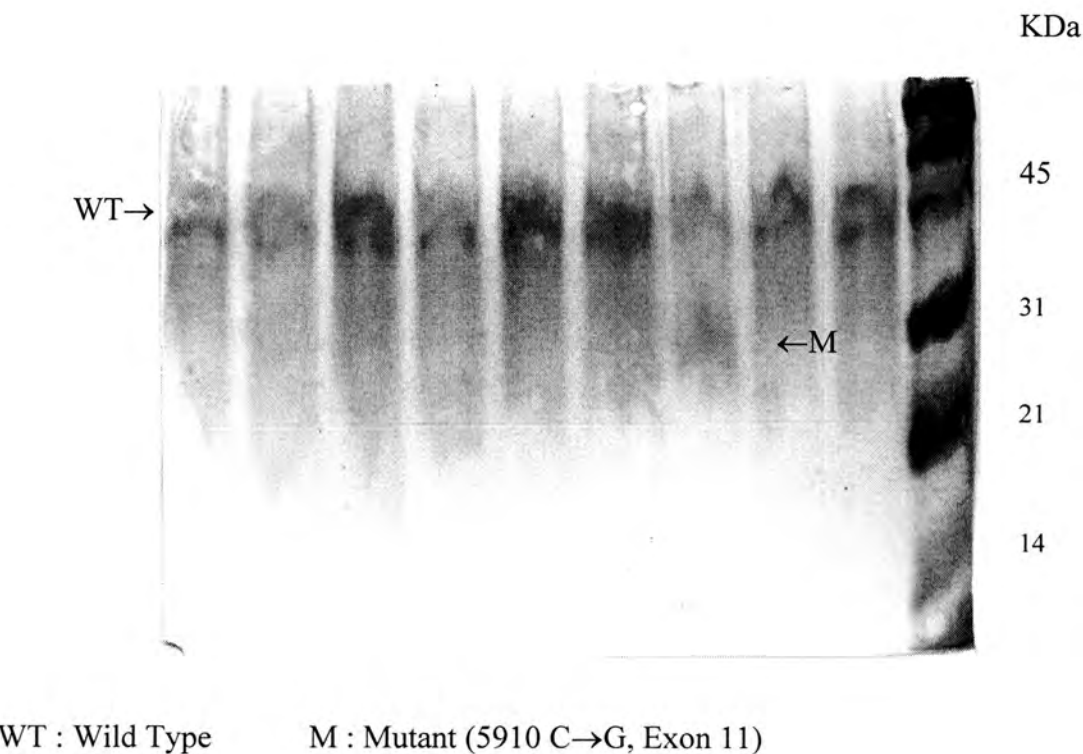
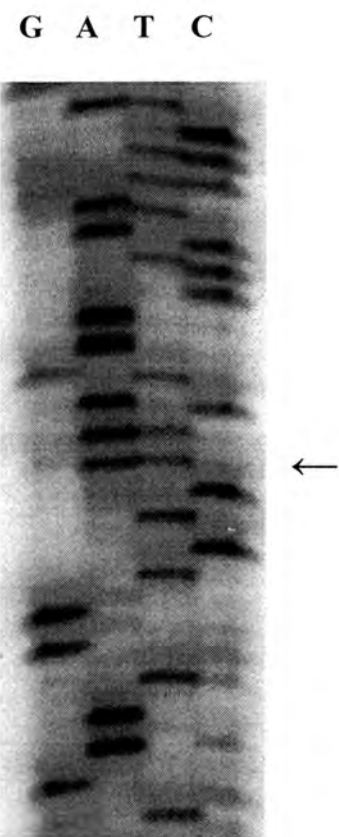


Figure 5.7

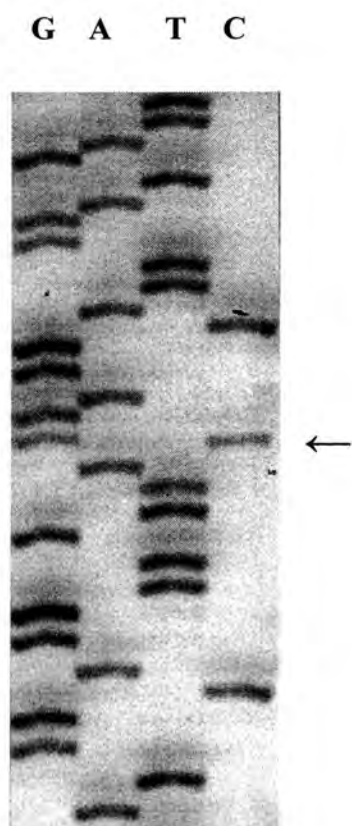
Cycle sequencing of PCR product containing BRCA2 1418 insTTAG mutation



5'-TGAATGGTCTCAACTAACCCCTTCA-3' Wild-type
5'-TGAATGGTCTCTTTAGAACTAACCCCT-3' Mutant

Figure 5·8

Cycle sequencing of PCR product containing BRCA2 5910 C→G mutation

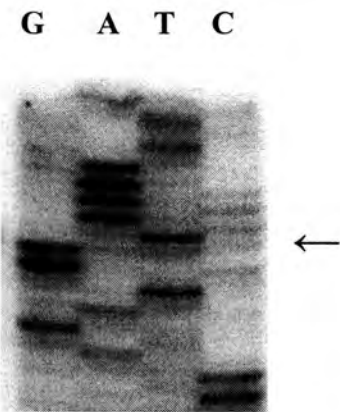


5'-ATGGCAGGTTGTTACGAGGCATTGGATGATT-3' Wild-type

5'-ATGGCAGGTTGTTAGGGAGGCATTGGATGATT-3' Mutant

Figure 5.9

Cycle sequencing of PCR product containing BRCA2 7165+2 delT mutation

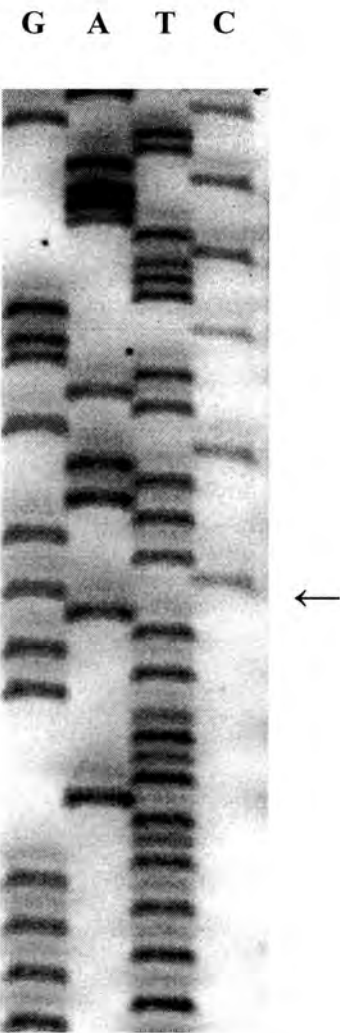


5'-CCAGATGgttaaaatt-3' Wild-type

5'-CCAGATGgttaaaatt-3' Mutant

Figure 5-10

Cycle sequencing of PCR product containing BRCA2 7830 del16 mutation

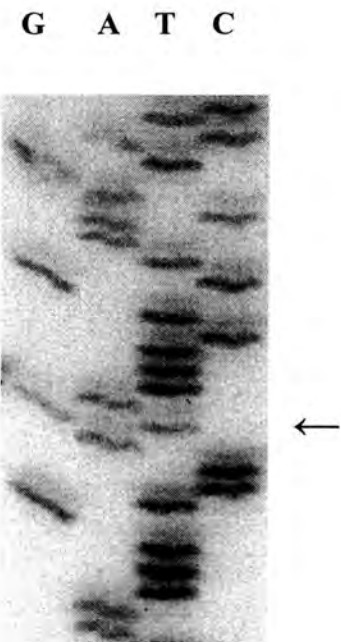


5'-gtgtgtgtttattttgtgtagGTGTTCTCATAAACAGCTGTATACGTA-3' Wild-type

5'-gtgtgtgtttattttgtgtagCTGTATACGTATGGCGTTTCTAAACAT-3' Mutant

Figure 5-11

Cycle sequencing of PCR product containing BRCA2 8560-1 G→T mutation



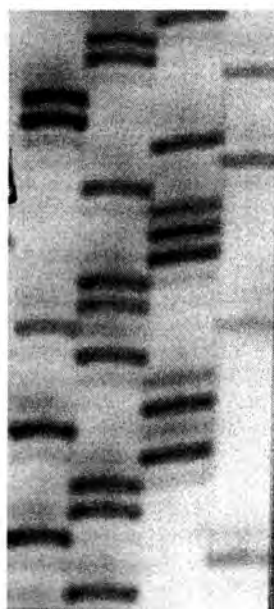
5'-aatttgtccagATTCTGCTAACAGTACTC-3' Wild-type

5'-aatttgtccatATTCTGCTAACAGTACTC-3' Mutant

Figure 5-12

Cycle sequencing of PCR product containing BRCA2 9522 C→G mutation

G A T C



5'-ACGAATGTTACAATTTACTGGCAAT-3' Wild-type

5'-ACGAATGTTAGAAATTTACTGGCAAT-3' Mutant

Figure 5-13

Cycle sequencing of PCR product containing BRCA2 9877-67 delTTAC mutation

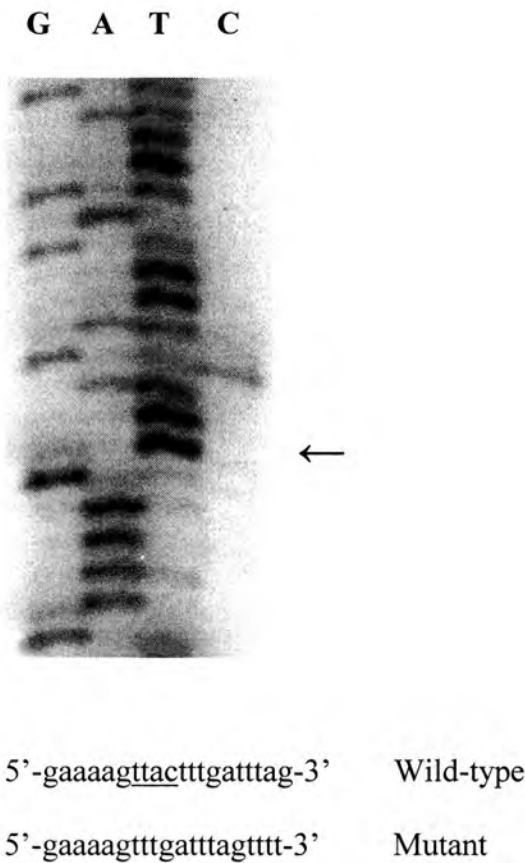


Figure 5.14

Family pedigree for MBC3
(BRCA2 8560-1 G→C)

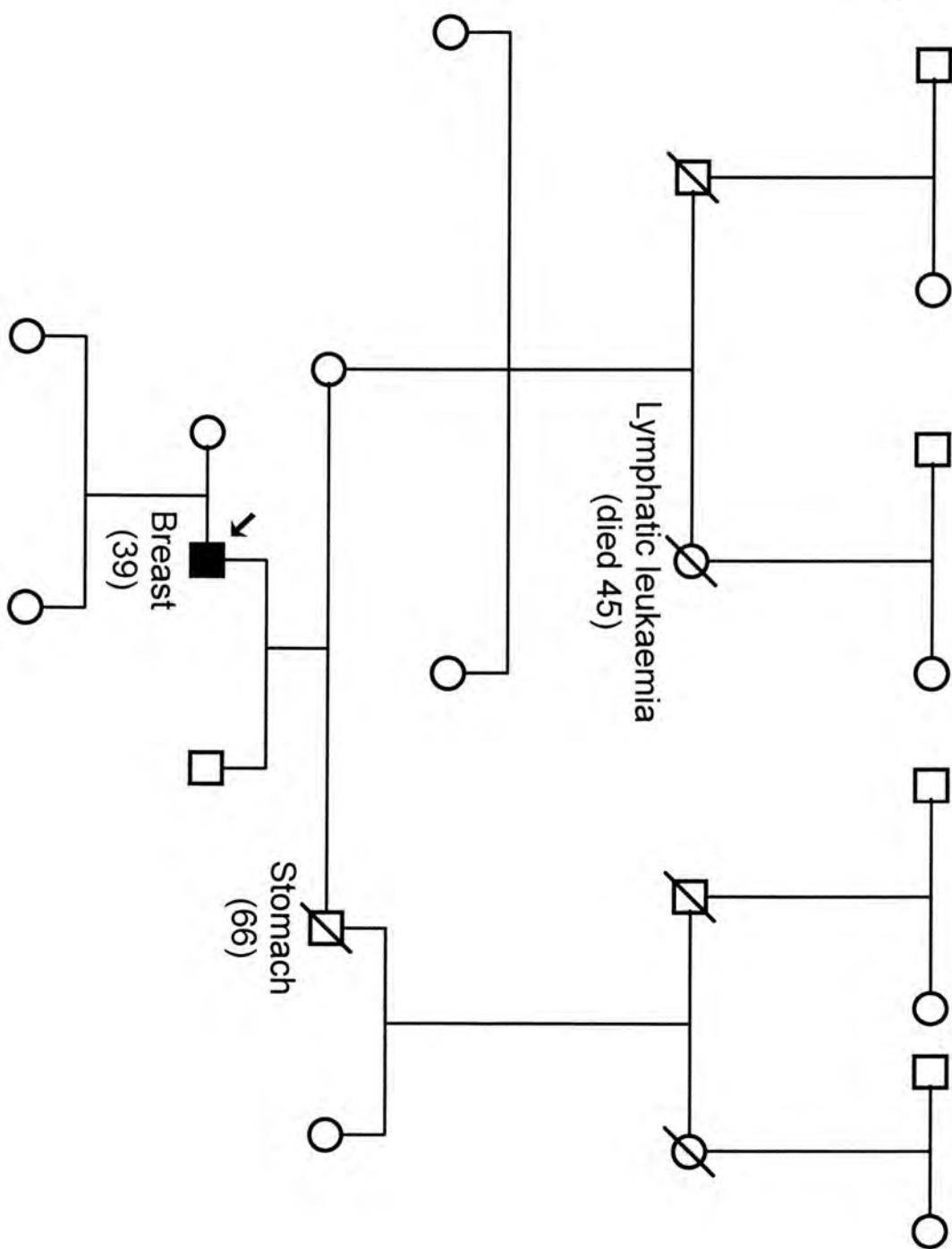


Figure 5-15

Family pedigree for MBC17

(BRCA2 9877-67 delTTAC)

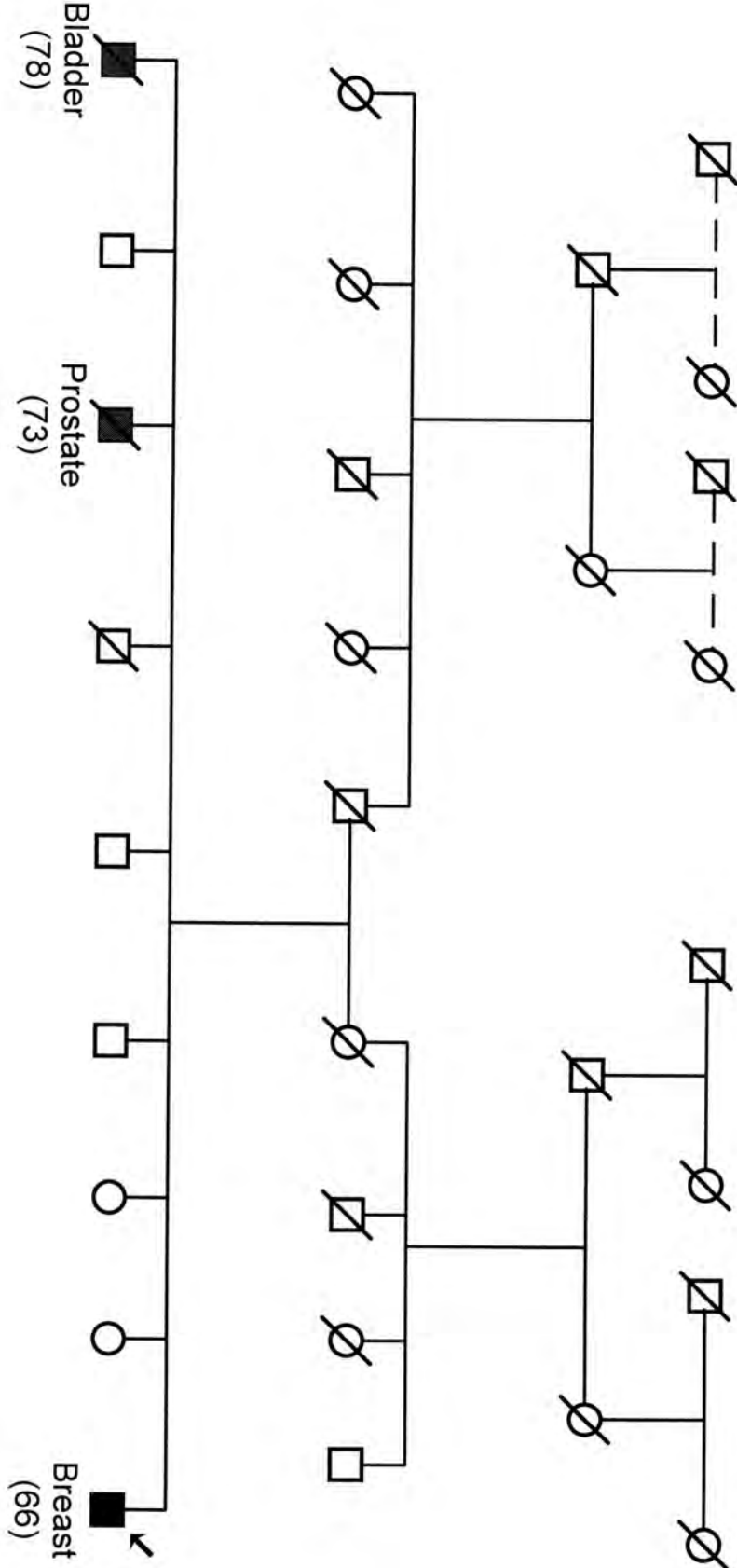


Figure 5-16

Family pedigree for MBC18

(BRCA2 1418 instTAG)

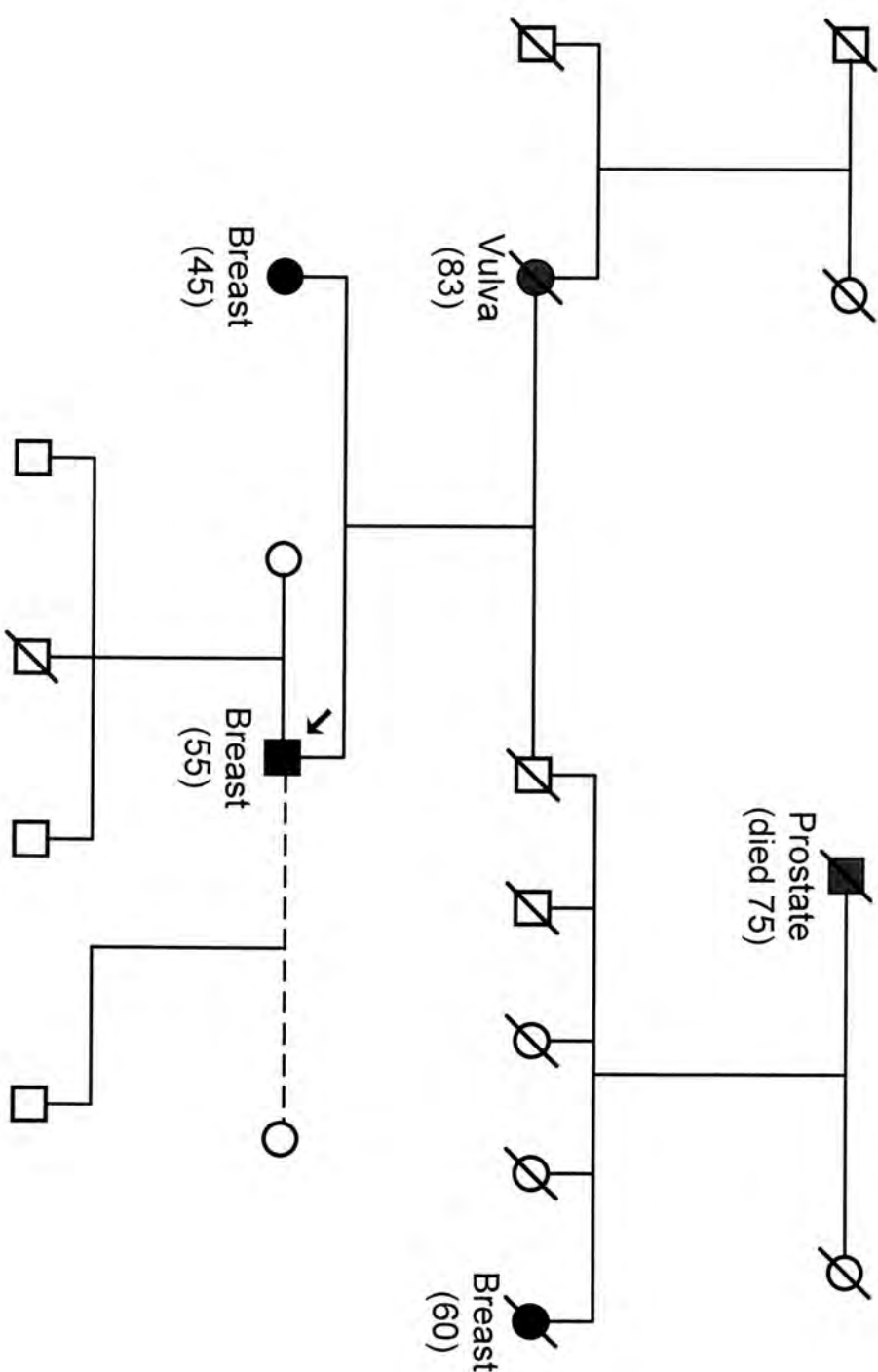


Figure 5.17

Family pedigree for MBC26

(BRCA2 7830 del16)

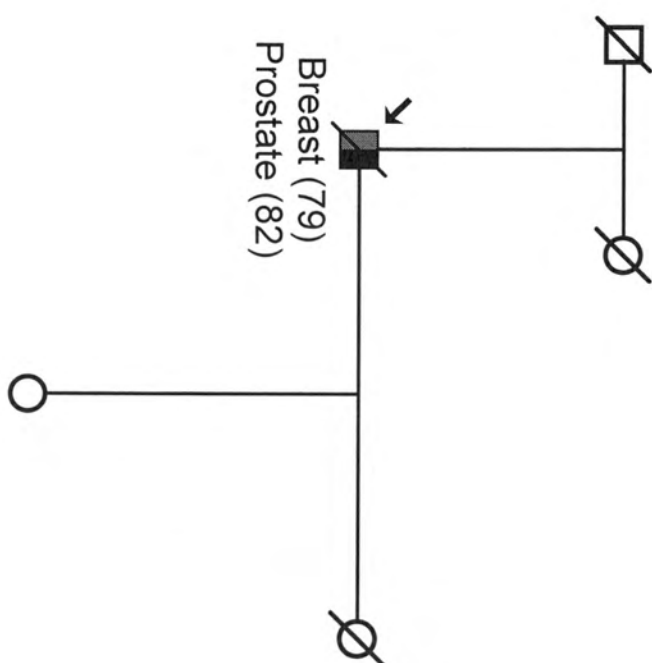
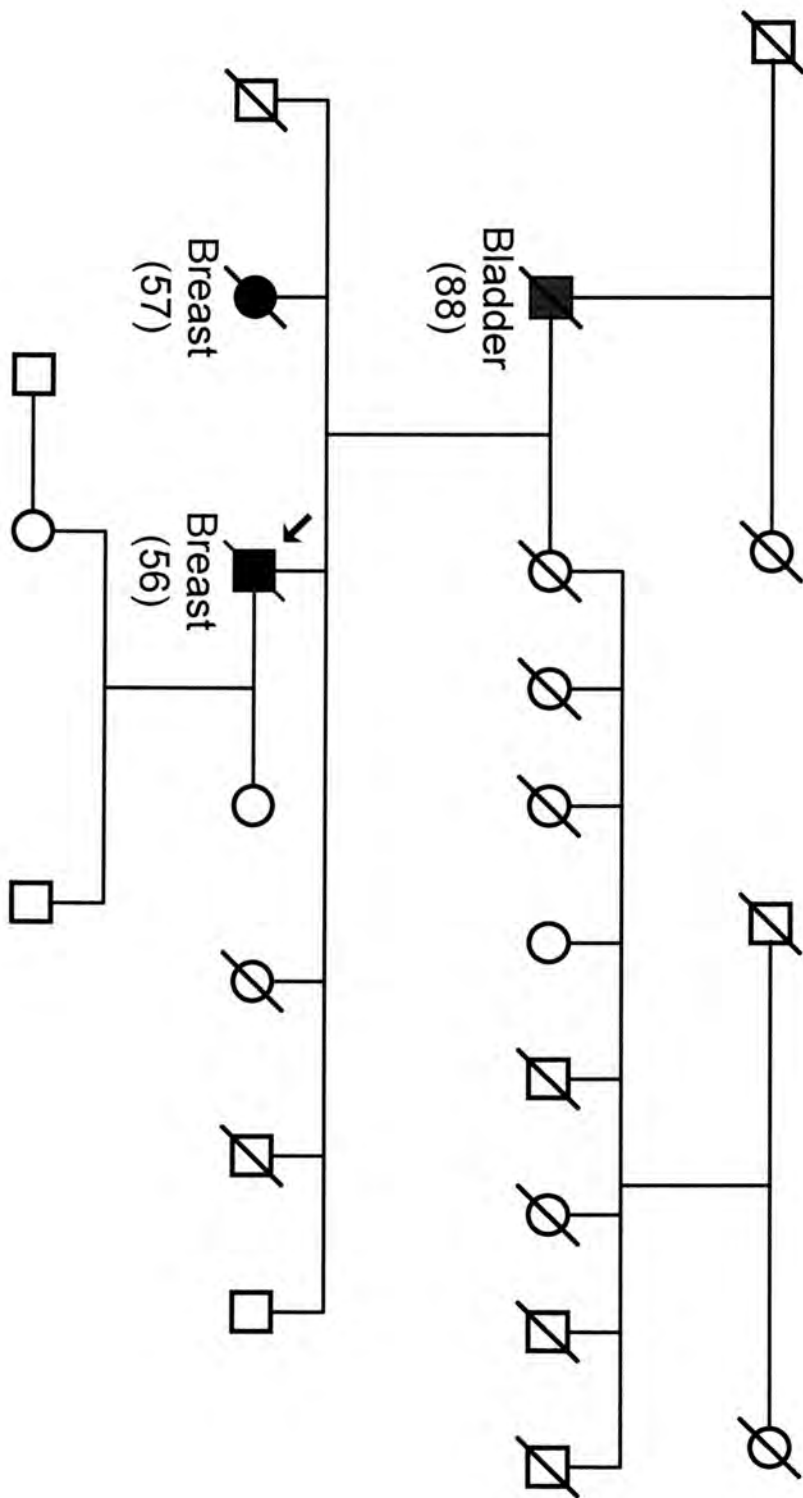


Figure 5-18

Family pedigree for MBC28

(9877-67 delTTAC)

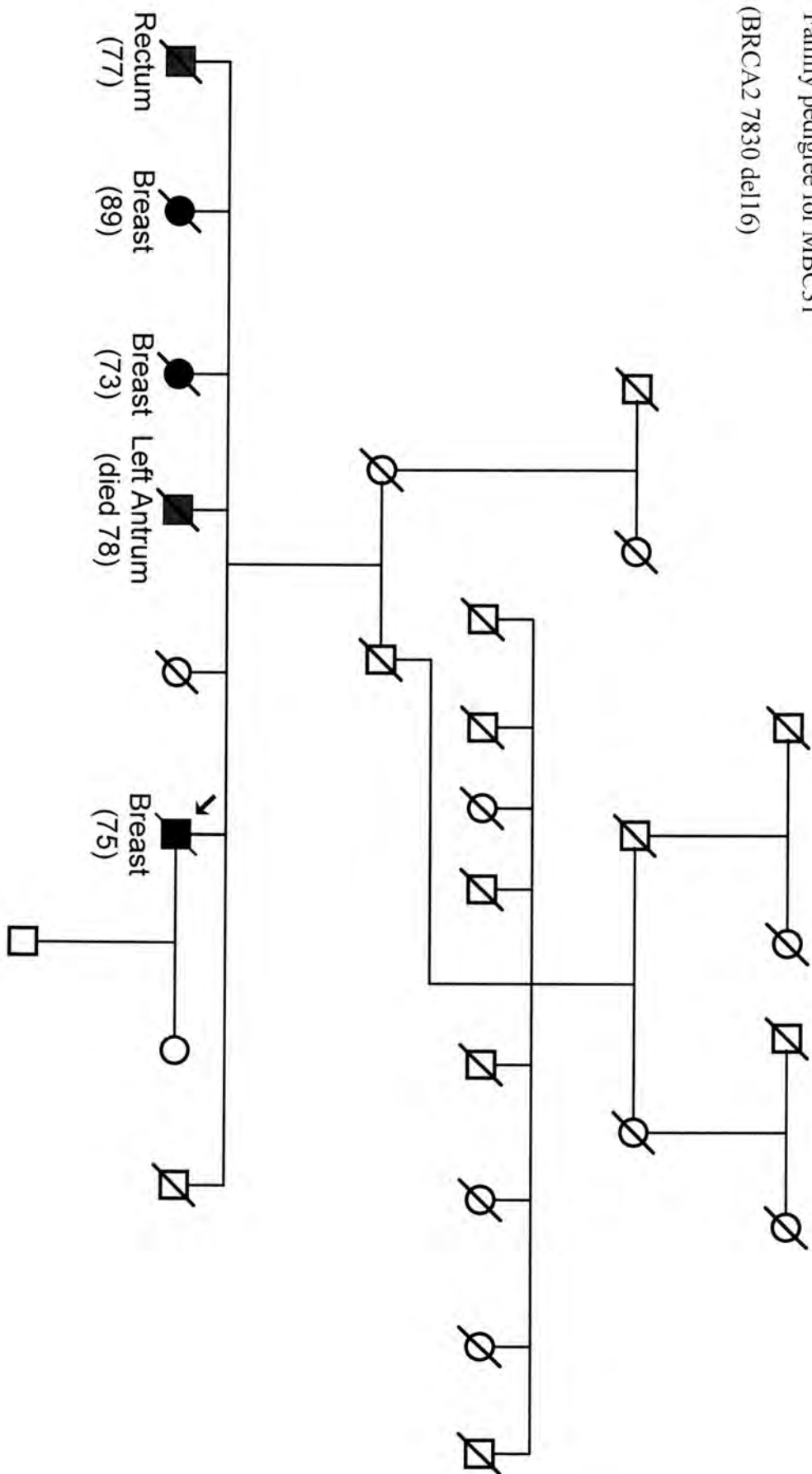


Family pedigree for MBC29

Pedigree chart showing four generations of a family. The chart includes symbols for males (squares) and females (circles). Affected individuals are marked with black symbols. Key affected individuals are labeled with their condition and age in parentheses: Liver (44), Brain (died 52), Lung (died 39), and Breast (71). The chart illustrates the inheritance pattern of a trait across four generations, with affected individuals appearing in every generation.

Figure 5-20

Family pedigree for MBC31
(BRCA2 7830 del16)



Family pedigree for MBC55

(BRCA2 7830 del16)

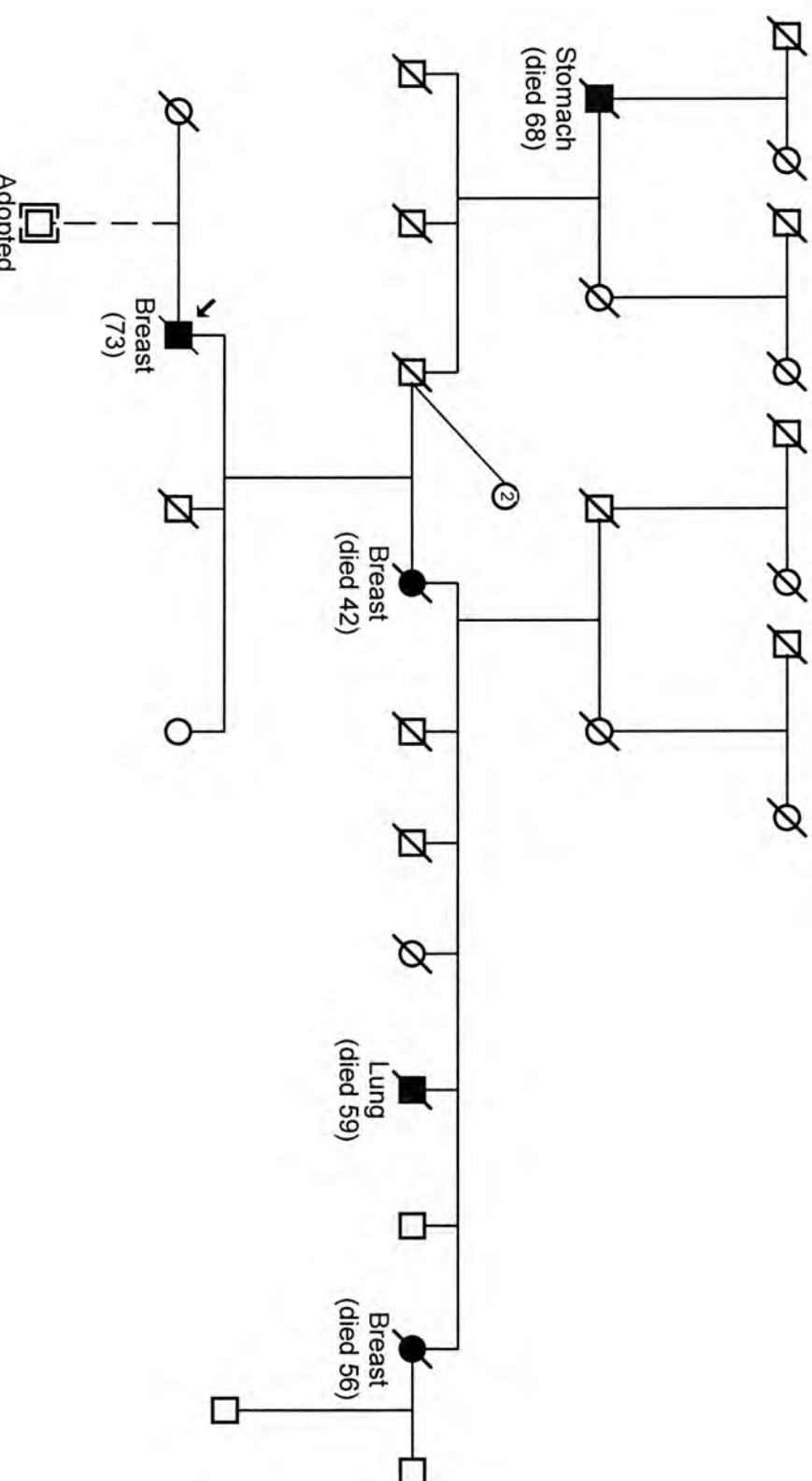


Figure 5.22

Family pedigree for MBC56

(BRCA2 7165+2 delT)

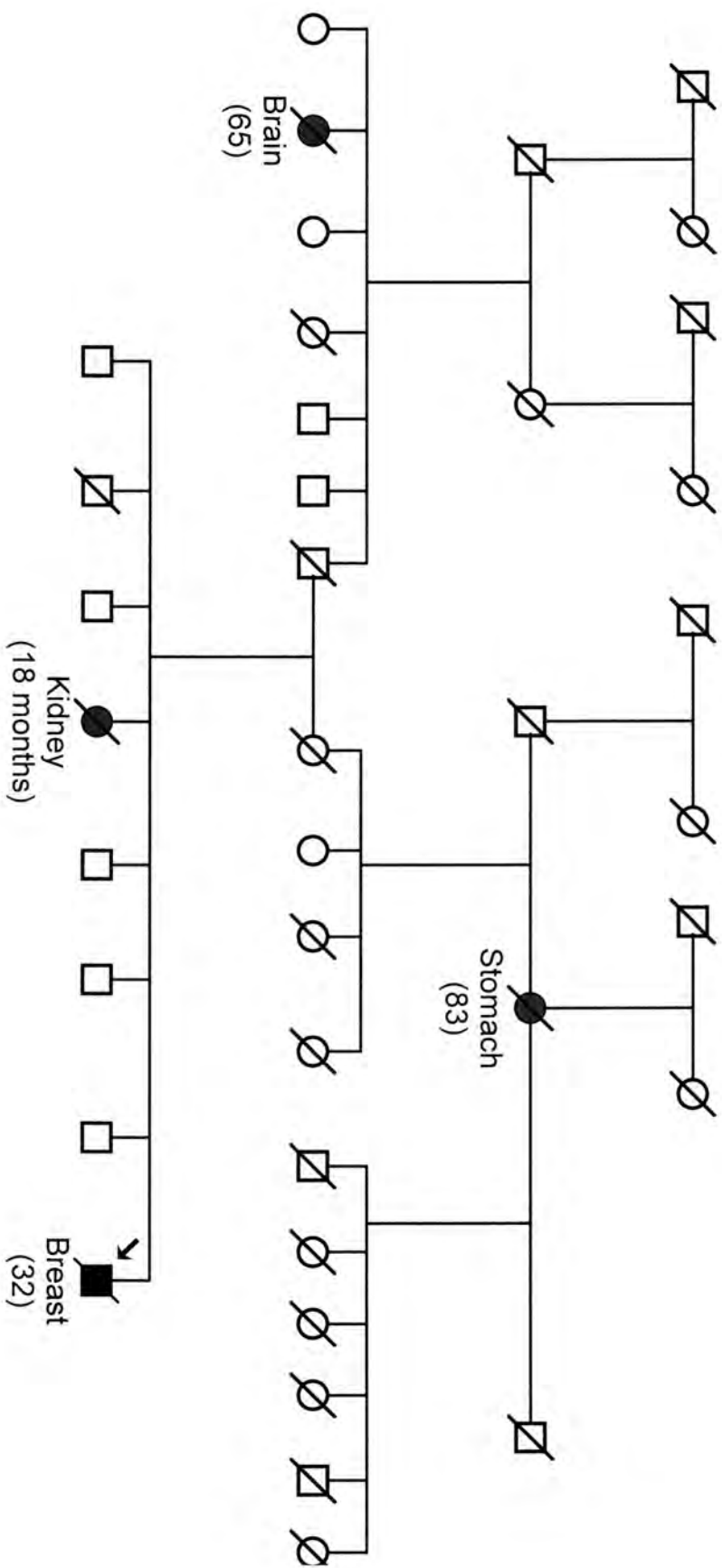


Figure 5.23

Family pedigree for MBC61

(BRCA2 7165+2 delT)

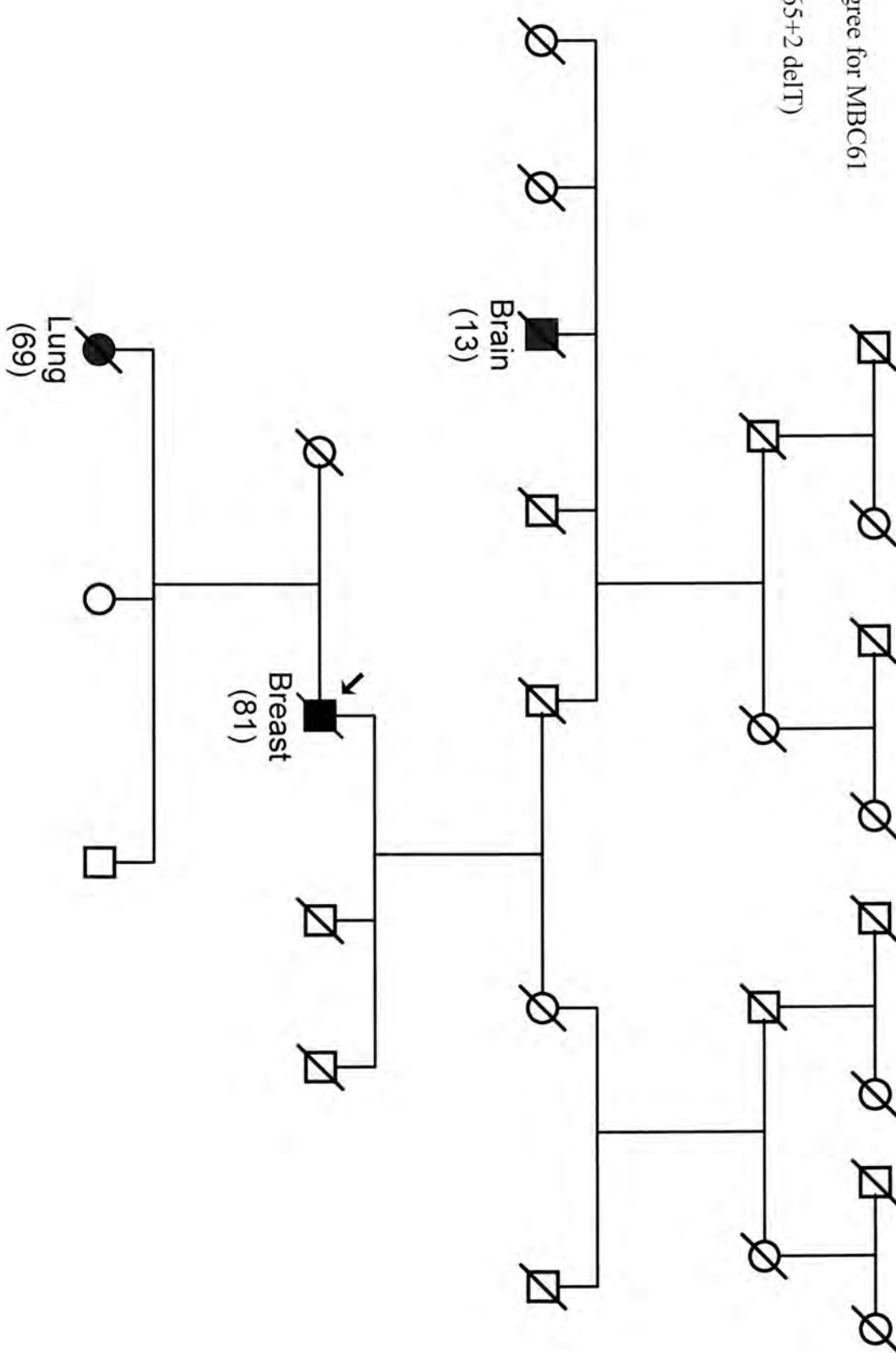


Figure 5.24

Family pedigree for MBC63

(BRCA2 5910 C→G)

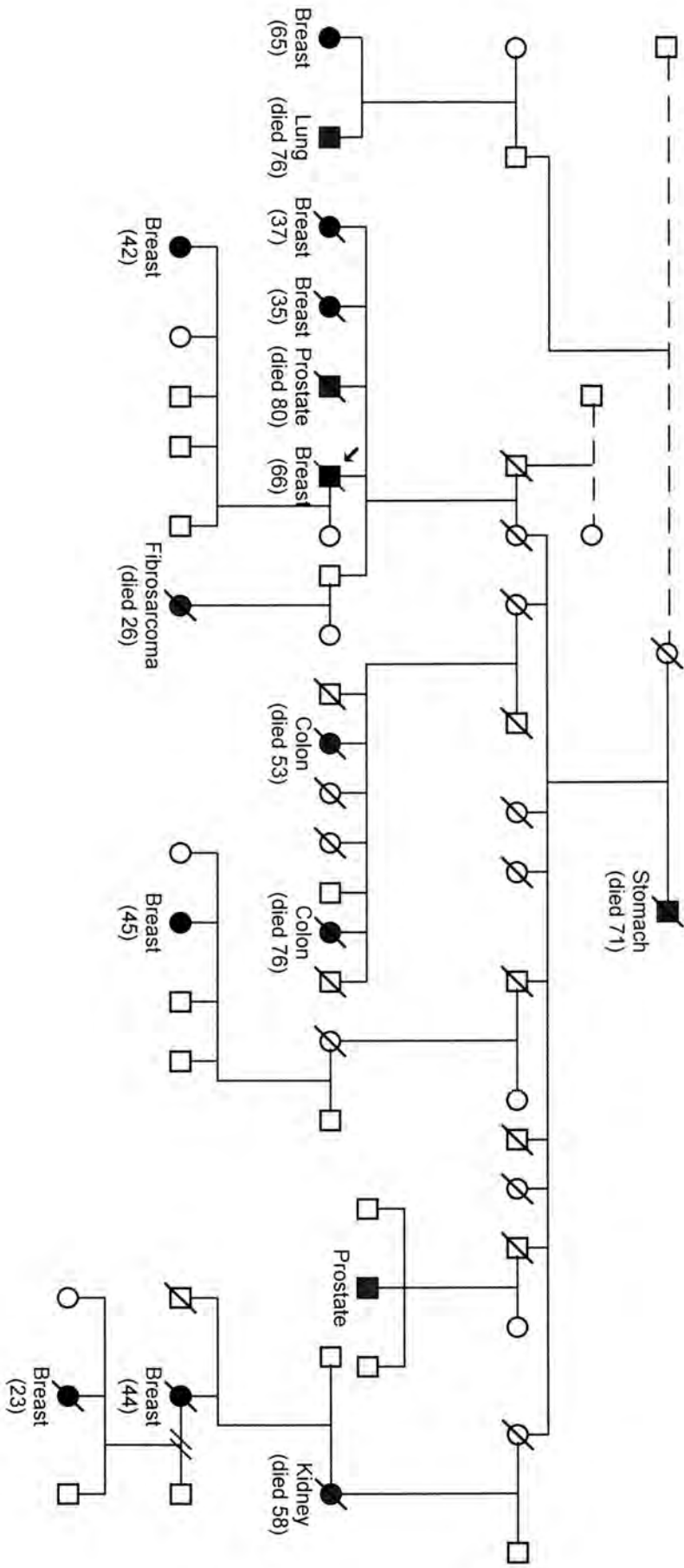


Figure 5.25

Family pedigree for MBC73

(BRCA2 9522 C→G)

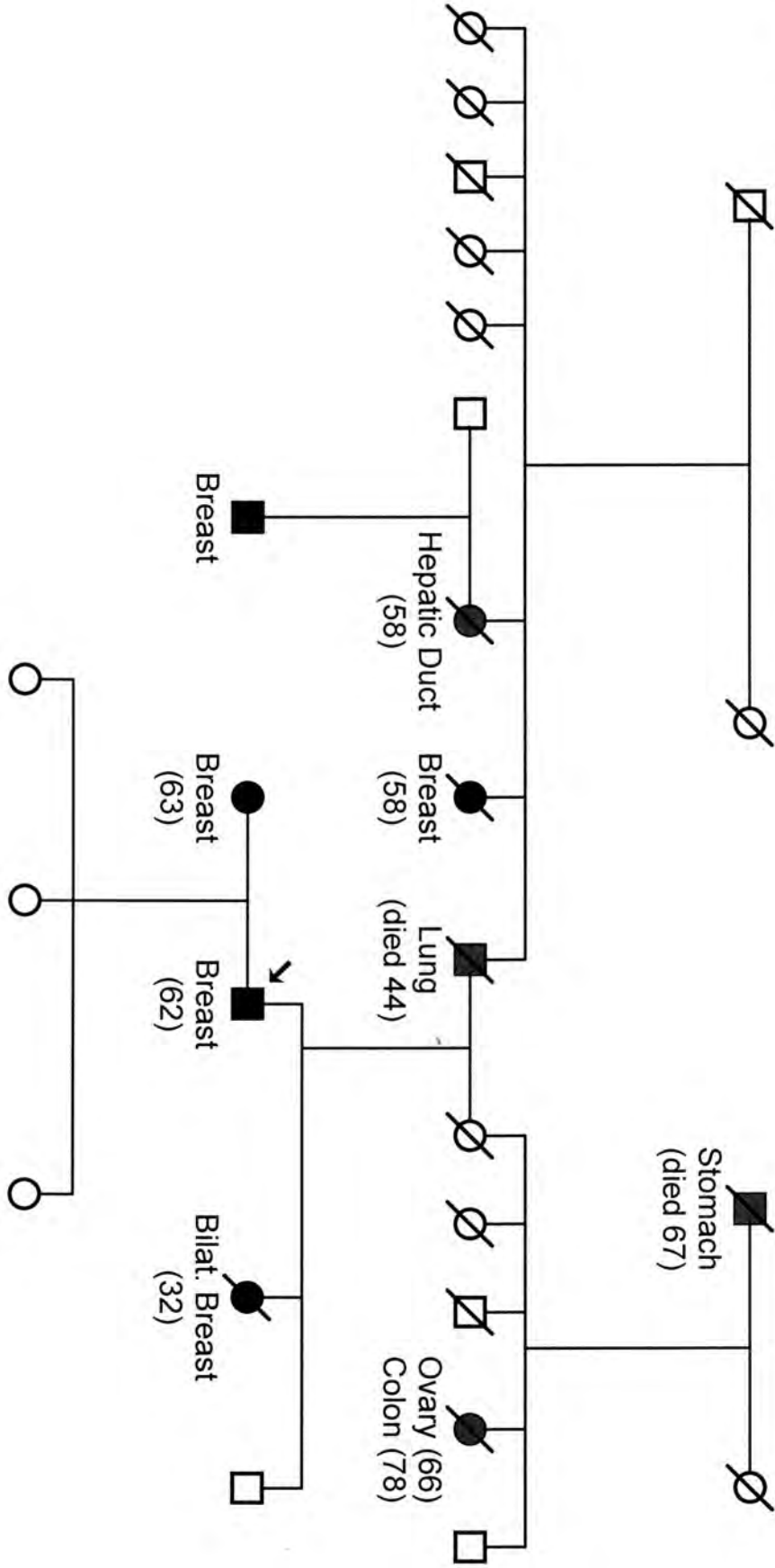
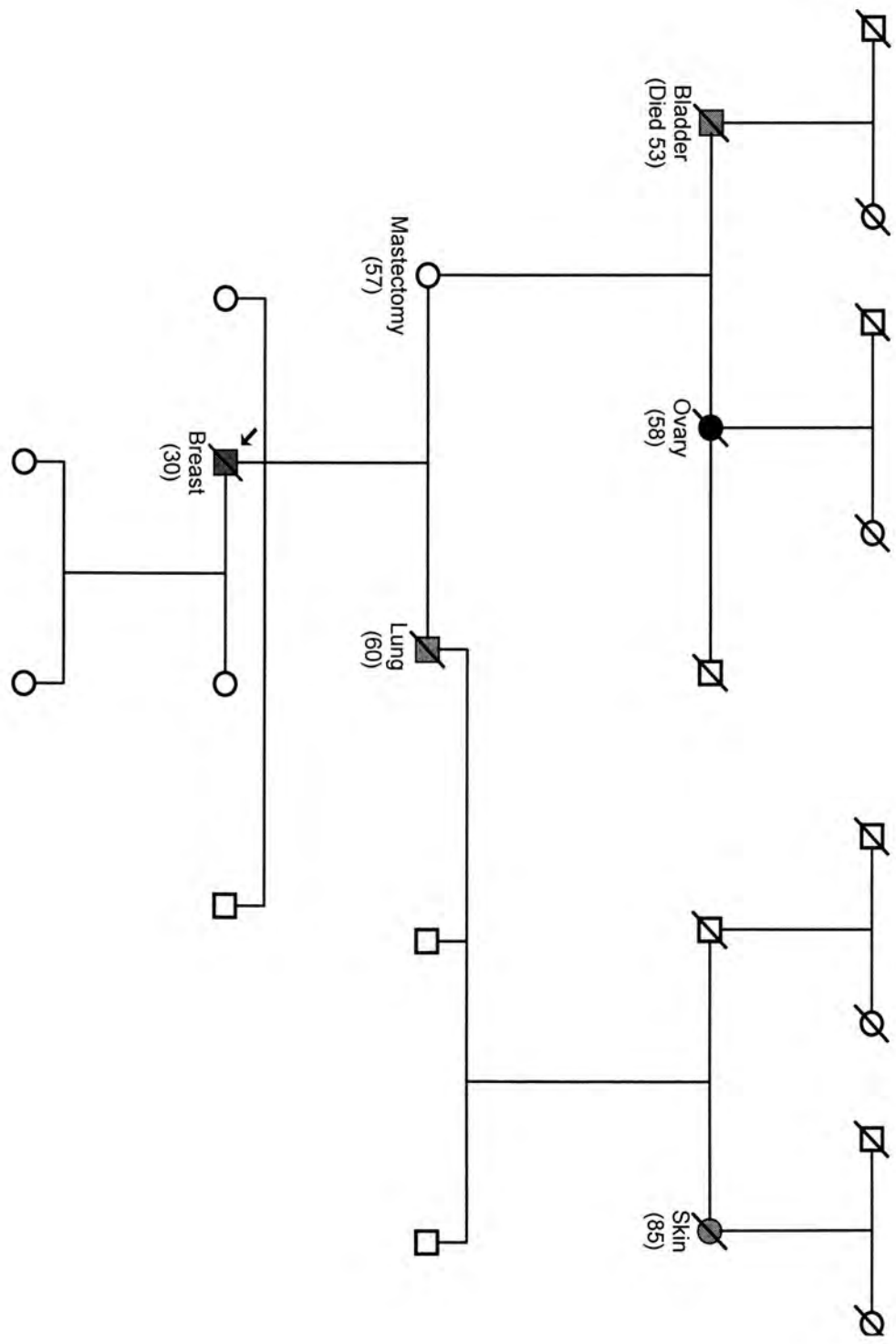


Figure 5-26

Family pedigree for MBC30

(No mutation detected)



Family pedigree for MBC33

The pedigree chart illustrates the family structure across three generations. The proband, a male with rectal cancer, is indicated by a shaded square in the second generation. The chart shows multiple marriages and the presence of several children in each generation, with some individuals marked as deceased by a diagonal line through their symbol.

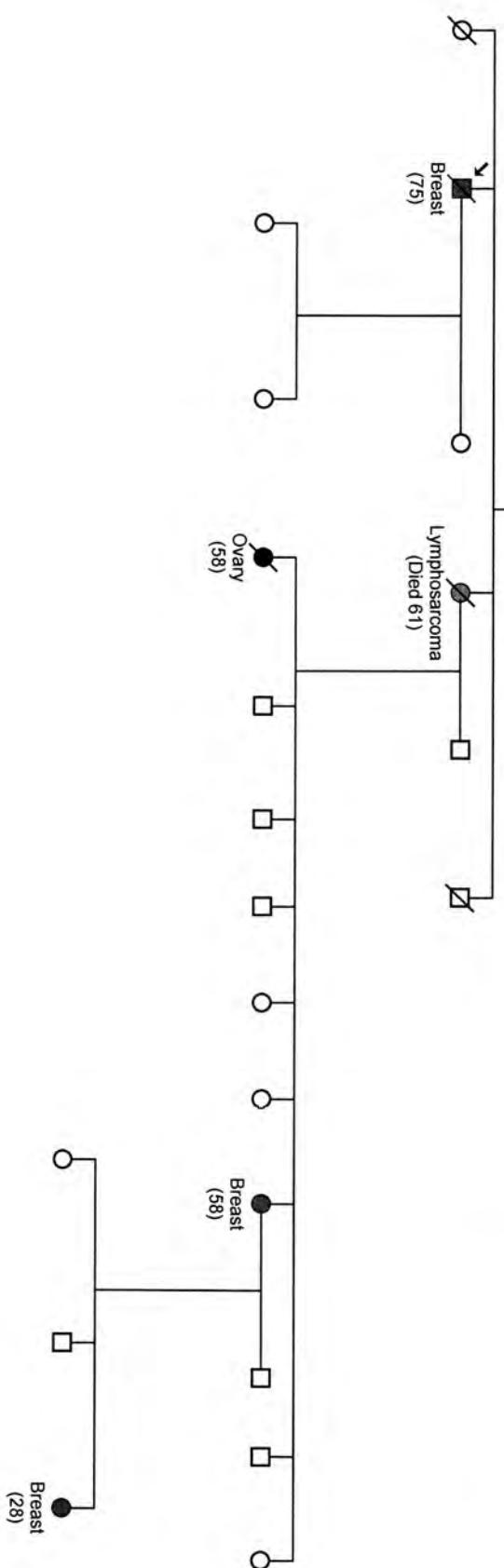


Figure 5.28

Family pedigree for MBC46
(No mutation detected)

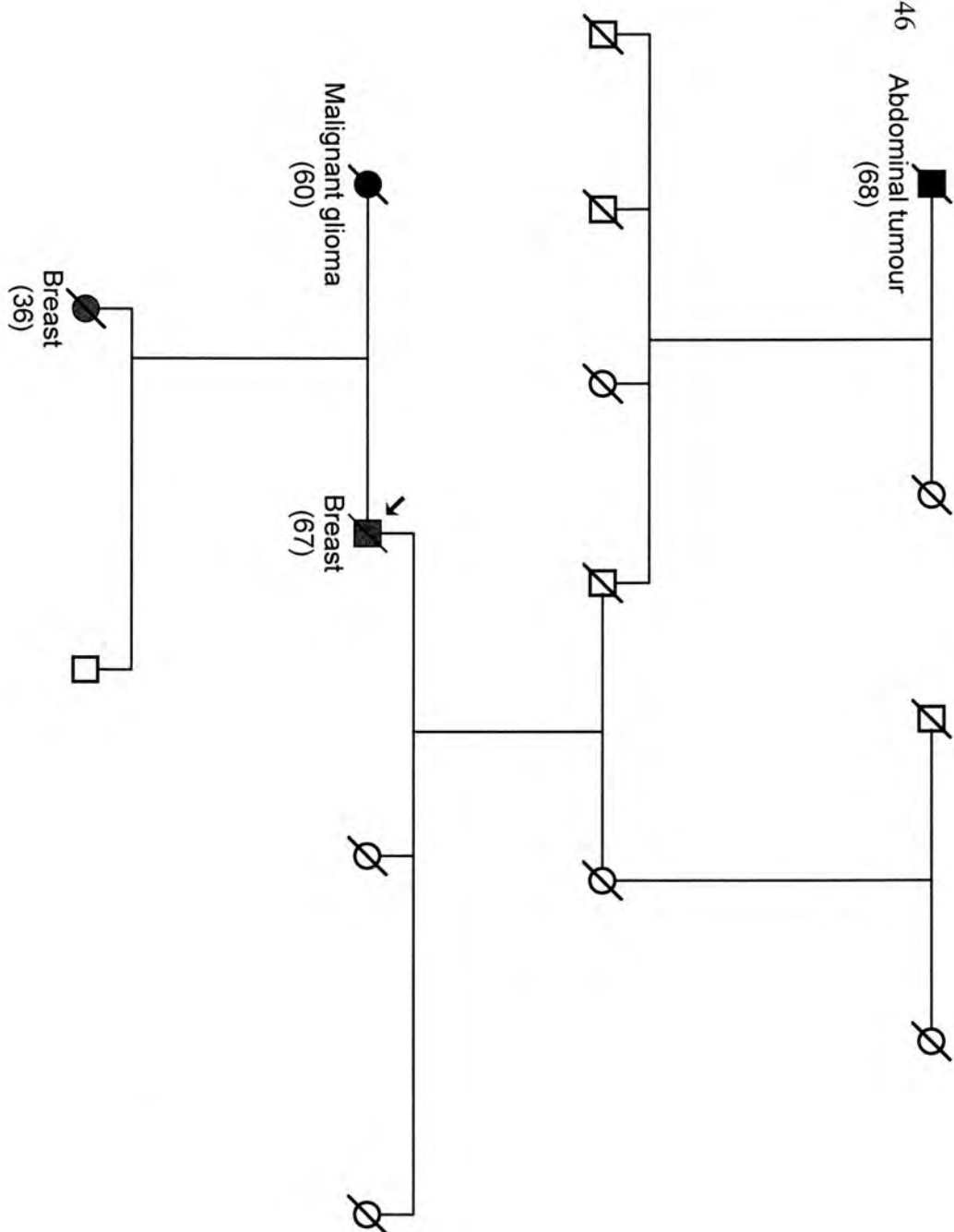
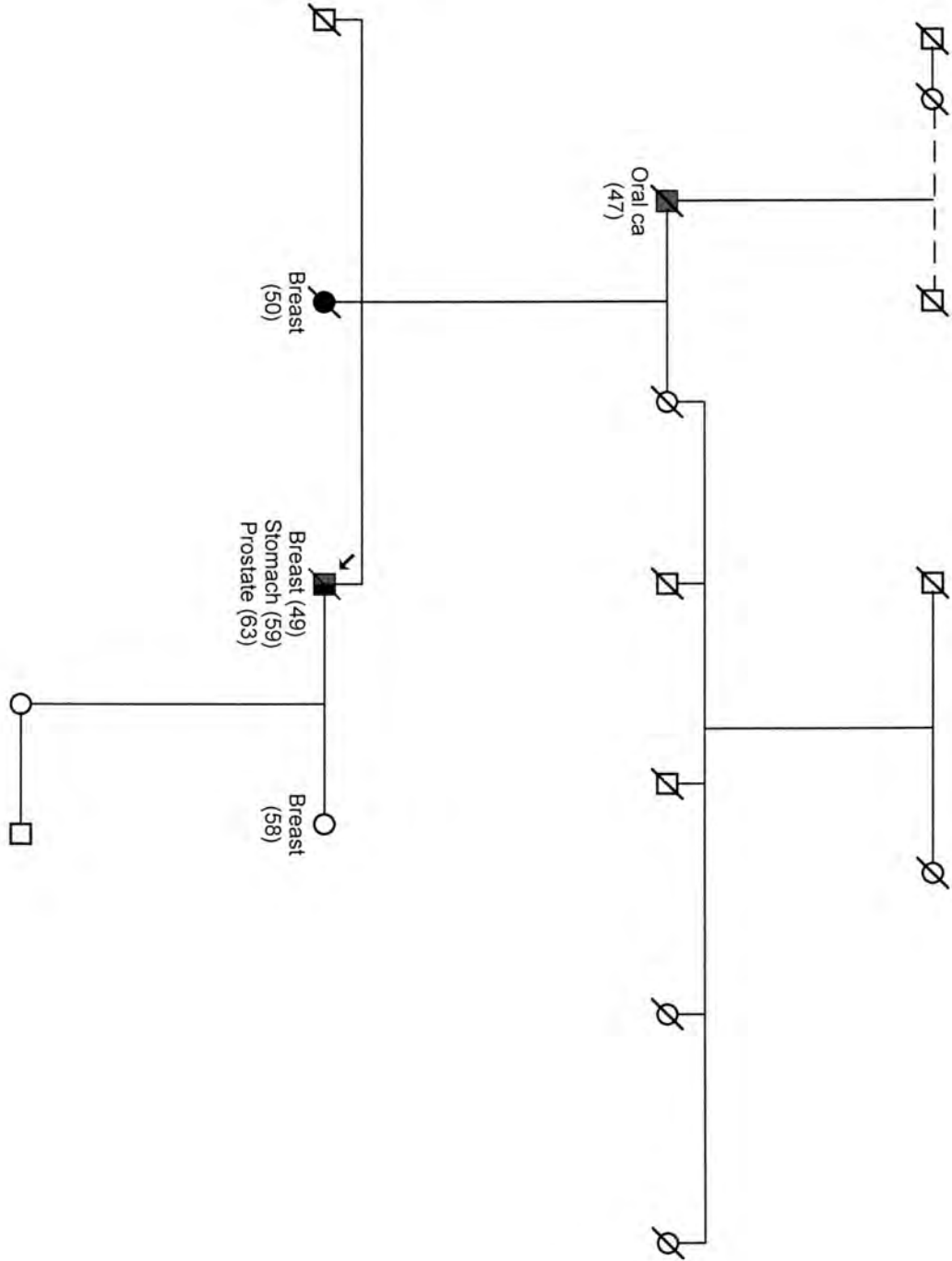


Figure 5-29

Family pedigree for MBC77
(No mutation detected)



Pedigrees for four of the males in whom no BRCA2 mutations were detected are shown in Figures 5·26 to 5·29. MBC30 developed breast cancer at the age of 30. His mother had breast cancer aged 57 and his maternal grandmother had ovarian cancer aged 58. MBC33 had a niece who developed ovarian cancer aged 58, another niece with breast cancer aged 58 and her daughter developed breast cancer aged 28. MBC46 had a daughter who developed breast cancer aged 36. MBC77 had breast, gastric and prostate cancer. His sister had breast cancer aged 50 and his father developed oral cancer aged 47.

Referring to the family pedigrees shown in Figures 5·14 to 5·25, the 12 male probands found to carry BRCA2 mutations had 35 female first-degree relatives in total. Of these, 9 (26%) had a history of breast cancer. Examination of the pedigrees for the 52 male probands in whom no BRCA2 mutations were detected, showed a total of 133 female first-degree relatives. Of these, 5 (4%) had a history of breast cancer.

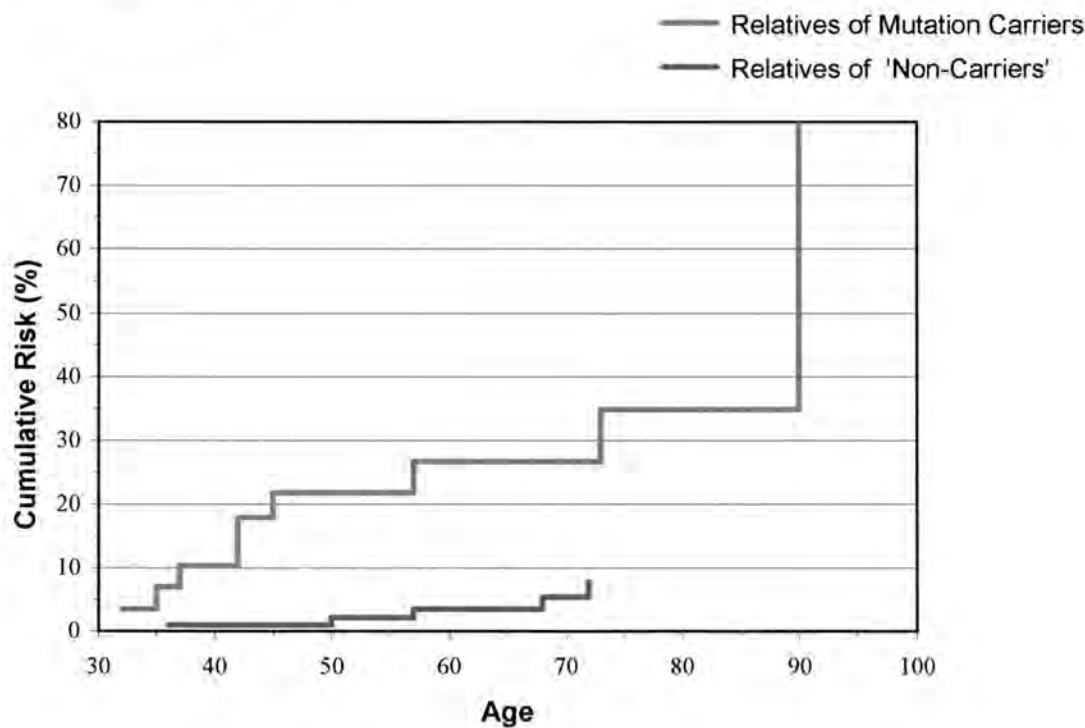
Figure 5·30 shows the estimated cumulative risks of breast cancer in female first-degree relatives of proven BRCA2 mutation carriers (indicated in red) and the relatives of those males not found to carry mutations (shown in blue). The curves were plotted using the Kaplan-Meier method (Bland & Altman, 1998).

The estimated cumulative risk of breast cancer is significantly higher in the group of first-degree female relatives of proven BRCA2 mutation carriers, compared to relatives of those males in whom no mutation was detected ($p=0.00002$, log-rank test). For first-degree female relatives of male breast cancer cases in whom a BRCA2 mutation has been detected, the estimated cumulative risk of breast cancer to age 73

is 34.8% (95% confidence interval 28.1% - 43.1%). However, if no BRCA2 mutation is detected in the male proband, then estimated cumulative risk of breast cancer in first-degree female relatives by age 72 is 7.8% (95% confidence interval 7.3% - 8.4%). This is similar to the risk of female breast cancer in the Scottish population as a whole, which is reported to be 7.9% to the age of 74 (Cancer Registration Statistics Scotland 1986 – 1995).

Figure 5-30

Estimated cumulative risks of breast cancer in female first-degree relatives of male breast cancer cases



Penetrance (the probability of cancer in a mutation carrier) can be estimated by calculating twice the age-specific cancer incidence in first-degree relatives of mutation carriers, minus the incidence in first-degree relatives of non-carriers

(Thorlacius et al, 1998). In our study the estimate of breast cancer penetrance for the BRCA2 mutations detected as a whole, in females by age 72-73, is therefore 61.7% (95% confidence interval 48.8% - 77.8%).

The numbers of other cases of different cancers among male and female first-degree relatives of the male probands are shown in Table 5.3. The numbers of these other cancers are small and there are therefore no apparent differences between relatives of carriers and non-carriers.

Table 5.3

	Cancers	Male Proband BRCA2 +ve	Male Proband BRCA2 –ve
Female First-degree Relatives	Breast	9 / 35	5 / 133
	Ovary	0 / 35 *	0 / 133 *
Male First-degree Relatives	Prostate	2 / 49	1 / 134
All First-degree Relatives	Pancreas	0 / 84	1 / 267
	Kidney	1 / 84	0 / 267
	Bladder	2 / 84	0 / 267
	Lung	2 / 84	5 / 267
	Stomach	1 / 84	4 / 267
	Colorectal	1 / 84	6 / 267
	Other	3 / 84	12 / 267

* Note, there were no cases of ovarian carcinoma among first-degree relatives, however there were a number of cases among second-degree relatives.

The clinical data are summarised in Table 5·4. The mean age at diagnosis of the group of 12 male breast cancer patients found to carry germline BRCA2 mutations was not significantly different from that for the group of 52 without mutations. There were also no significant differences between the two groups in terms of the histological classification of tumours, disease stage at presentation or risk of loco-regional relapse within 5 years.

Table 5·4

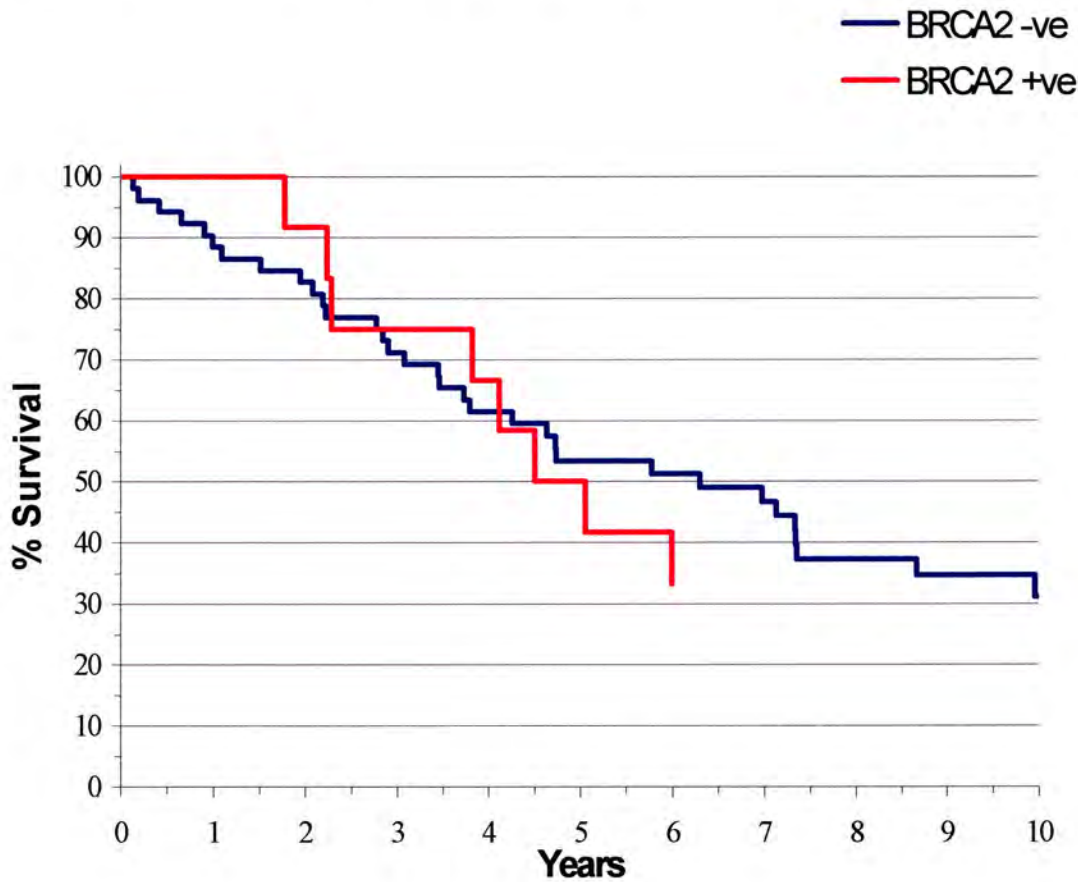
Clinical data for the male breast cancer cases

		BRCA2 -ve	BRCA2 +ve	p
Mean Age (Range)		65.4 (26-85)	63.3 (32-81)	0.676 (t test)
Histology	No special type	39	9	0.802 (χ^2 test)
	Special type	6	1	
	None	5	2	
	Not known	2	0	
Stage at presentation	I	11	3	0.894 (χ^2 test)
	II	20	4	
	III	13	4	
	IV	5	1	
	Not known	3	0	
Loco-regional relapse at 5 years		38.9%	50.0%	0.305 (log rank test)

Kaplan-Meier estimates of the cumulative survival for both groups are shown in Figure 5-31. Although the curves appear to diverge after four years, there was no statistically significant difference between the two groups ($p=0.449$, log rank test).

Figure 5-31

Estimates of cumulative survival for male breast cancer cases



5.3 CYP17

The different alleles of the CYP17 gene as demonstrated by agarose gel electrophoresis are shown in Figure 5·32. The results showing the genotyping of the cases and controls are shown in Table 5·5. The results show that a C allele is found significantly more frequently in male breast cancer patients than in male controls ($\chi^2=4.308$, 1 d.f., $p=0.038$). The odds ratio for the risk of a male with a C allele developing breast cancer is calculated as 2.10 (95% confidence interval 1.04-4.27). There were no significant differences between female breast cancer patients and female controls ($\chi^2=1.895$, 1 d.f., $p=0.169$; odds ratio=0.56, 95% confidence interval 0.25-1.28). The distribution of alleles in both control groups is in Hardy-Weinberg equilibrium (Table 5·6).

Figure 5·32

Different alleles of the CYP17 gene demonstrated by agarose gel electrophoresis

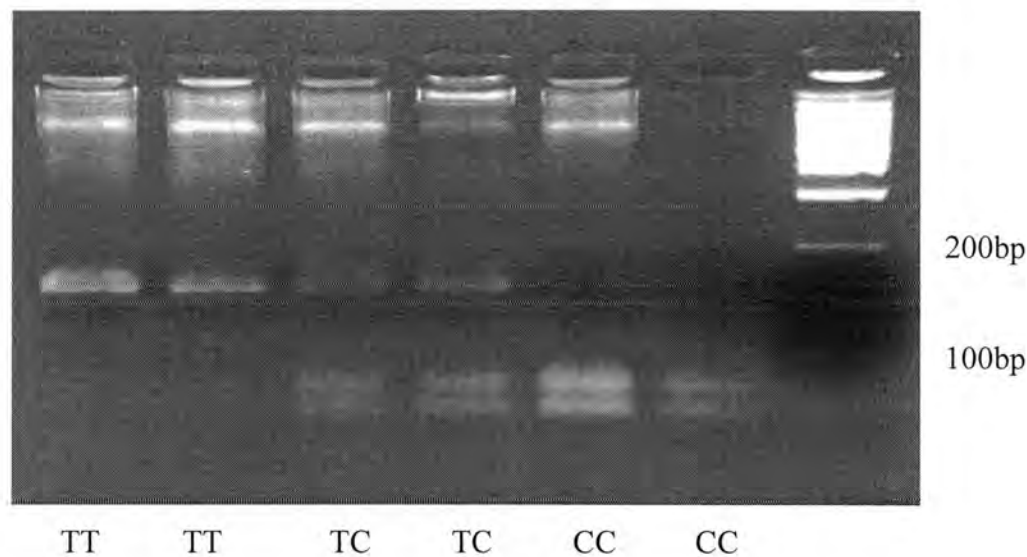


Table 5-5

CYP17 Genotype Frequencies Among Breast Cancer Patients and Controls

	Males		Females	
Genotype	Cases (n=64)	Controls (n=81)	Cases (n=39)	Controls (n=58)
TT	17 (26.6%)	35 (43.2%)	21 (53.8%)	23 (39.7%)
TC	42 (65.6%)	39 (48.1%)	13 (33.3%)	28 (48.3%)
CC	5 (7.8%)	7 (8.6%)	5 (12.8%)	7 (12.1%)
TC / CC	47 (73.4%)	46 (56.8%)	18 (46.2%)	35 (60.3%)

Table 5-6

CYP17 Genotype Frequencies In Controls and Expected Frequencies Under Hardy-Weinberg Equilibrium

	Male Controls		Female Controls	
	Observed	Expected	Observed	Expected
TT	35	36.7	23	23.6
TC	39	35.7	28	26.8
CC	7	8.67	7	7.60

($\chi^2=0.705$, 2 d.f., p=0.703)

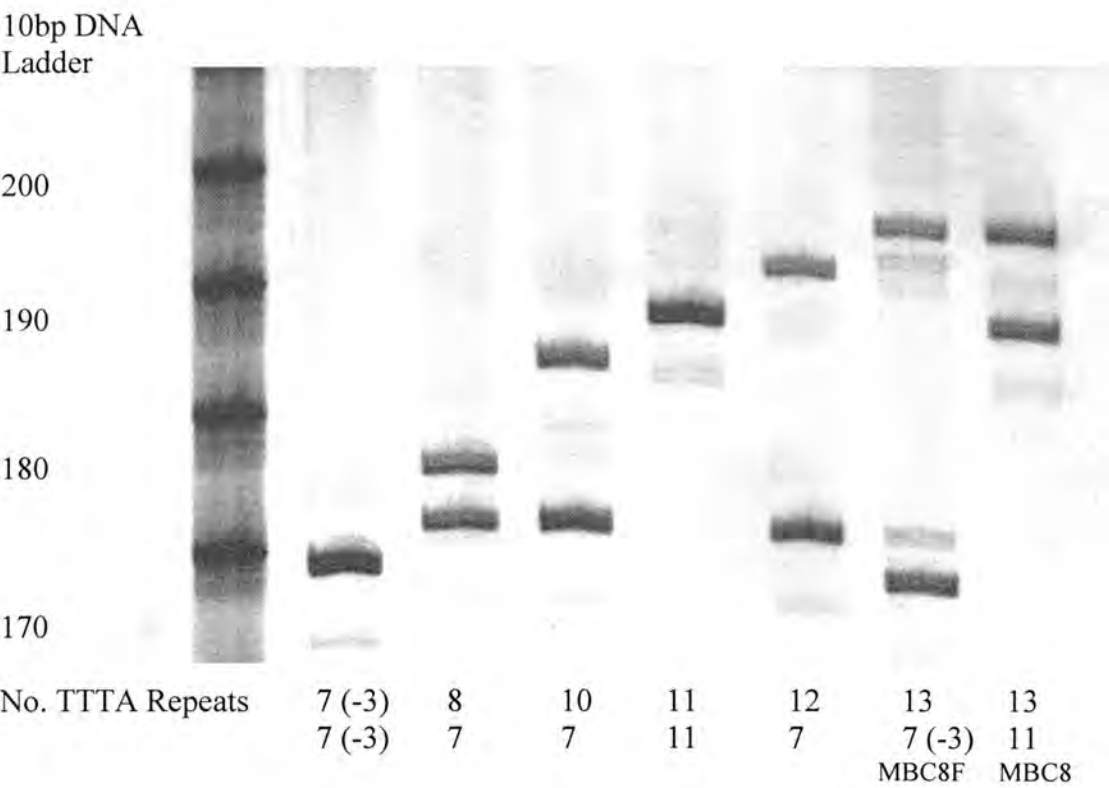
($\chi^2=0.116$, 2 d.f., p=0.943)

5.4 CYP19

PCR was unsuccessful with 10 of the DNA samples derived from archival wax-embedded tissue sections, giving a total of 54 case samples (108 alleles) analysed. Seven different alleles of the CYP19 gene were detected (Figure 5·33).

Figure 5·33

Examples of the seven different alleles of the CYP19 gene found in male breast cancer patients and controls.



The allele distribution of the CYP19 gene in male breast cancer patients and controls is shown in Table 5·7. There were no significant differences in the distribution of alleles between cases and controls ($p=0.838$). We have found two different alleles containing 7 TTTA repeats (corresponding to PCR products of 168bp and 171bp in

length). Cycle sequencing of these alleles revealed that the shorter allele, designated (TTTA)₇₋₃, had a 3-bp (CTT) deletion 50bp upstream from the 5' end of the TTTA repeat sequence (Figure 5-34). One of the male breast cancer patients (MBC8) had an allele containing 13 repeats. A blood sample was obtained from his father (MBC8F), who had not had breast cancer. Analysis of DNA extracted from this showed the same (TTTA)₁₃ allele.

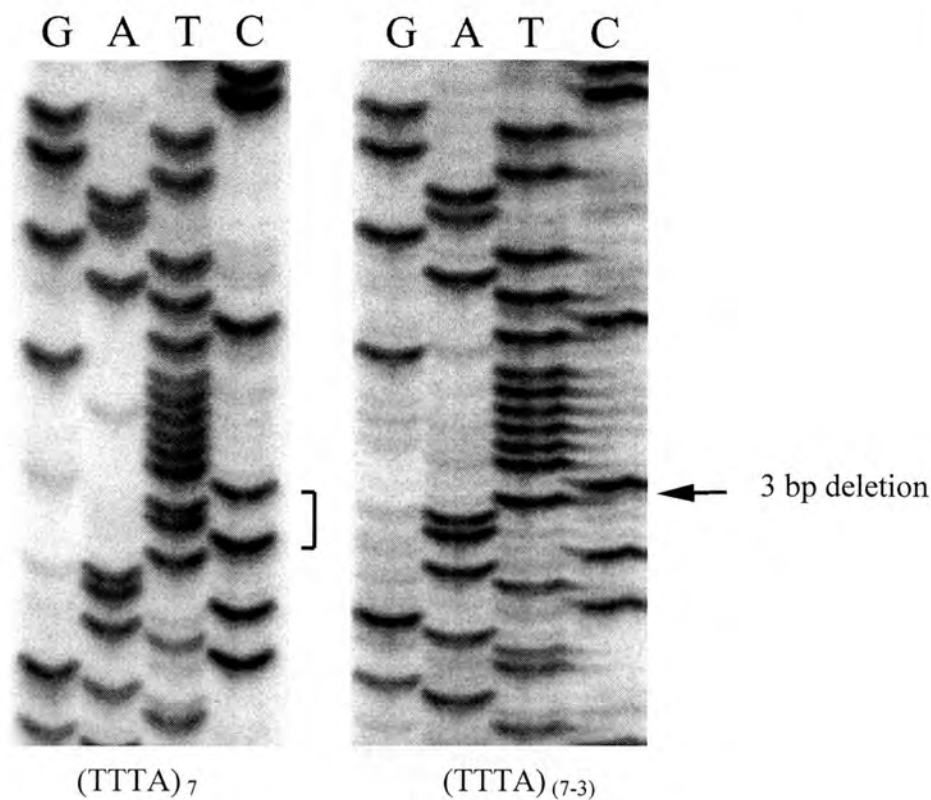
Table 5-7

Distribution of alleles of the CYP19 gene in male breast cancer patients and controls

Allele	PCR Product Length (bp)	Cases (n=108)	Controls (n=158)	Odds Ratio & 95% C.I.
(TTTA) ₍₇₋₃₎	168	35 (32.4%)	48 (30.4%)	1.10 (0.649 - 1.86)
(TTTA) ₇	171	20 (18.5%)	30 (19.0%)	0.970 (0.518 - 1.82)
(TTTA) ₈	175	11 (10.2%)	16 (10.1%)	1.01 (0.448 - 2.26)
(TTTA) ₁₀	183	1 (0.93%)	2 (1.3%)	0.729 (0.0653 - 8.14)
(TTTA) ₁₁	187	36 (33.3%)	57 (36.1%)	0.886 (0.529 - 1.48)
(TTTA) ₁₂	191	4 (3.7%)	5 (3.2%)	1.18 (0.309 - 4.49)
(TTTA) ₁₃	195	1 (0.93%)	0	-
Heterozygosity		79.6%	79.7%	

Figure 5.34

Sequencing of part of the (TTTA)₇ and (TTTA)₍₇₋₃₎ alleles of the CYP19 gene.



5'- TTAGTTAGCTACAATCTTCTTTTTTGTCTATGAATGTGC - 3'

5.5 Oestrogen Receptor Gene

PCR was unsuccessful with the DNA from one case (intron 1 and exon 2) and one control (intron 1 only). Different alleles of intron 1 and exon 2 demonstrated by agarose gel electrophoresis are shown in figures 5·35 and 5·36 respectively. The distribution of intron 1 alleles is shown in Table 5·8 and the distribution of exon 2 alleles in Table 5·9.

Figure 5·35

The different alleles of intron 1 of the oestrogen receptor gene on agarose gel electrophoresis

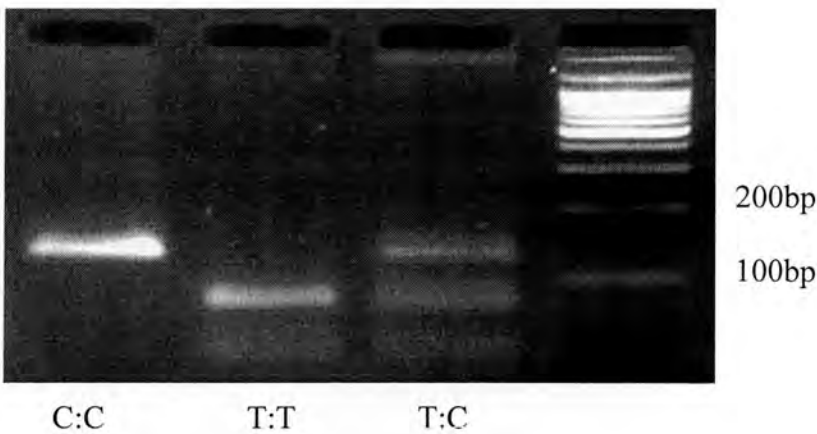


Figure 5.36

The different alleles of exon 2 of the oestrogen receptor gene on agarose gel electrophoresis

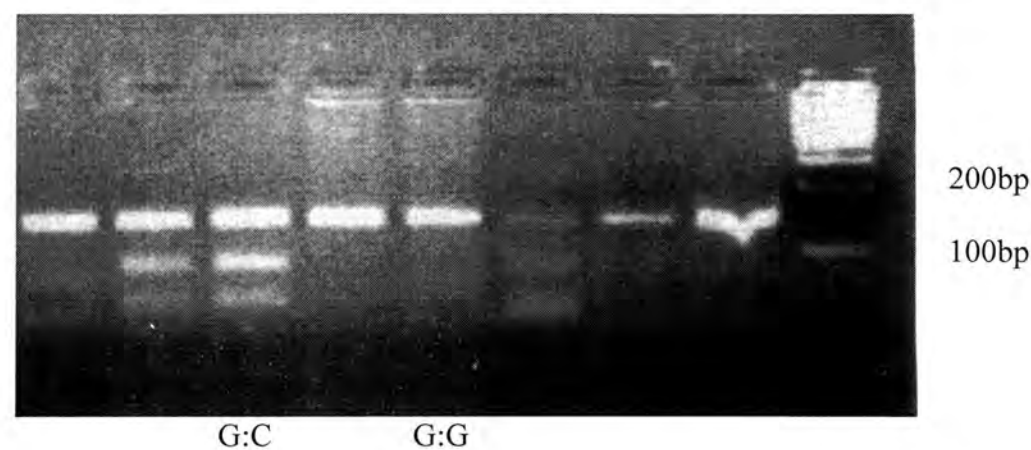


Table 5.8

The distribution of alleles of oestrogen receptor gene intron 1

Intron 1 Allele (PvuII)	Cases	Controls
TT	14	21
TC	38	38
CC	11	17
Total	63	76

Taking TC and CC together, $\chi^2=0.54$ (1 df), $p=0.464$

The odds ratio for the risk of breast cancer in a male with a polymorphic allele in intron 1 is 1.34 (95% C.I. 0.614 to 2.91).

Table 5·9

The distribution of alleles of oestrogen receptor gene exon 2

Exon 2 Allele (BstU1)	Cases	Controls
GG	46	67
GC	16	10
CC	1	0
Total	63	77

17 / 63 (27%) of males with breast cancer carry the polymorphic allele in exon 2 compared to 10 / 77 (13%) of controls. This difference was statistically significant ($\chi^2=4\cdot361$, 1 d.f., $p=0\cdot0368$). The odds ratio for the risk of breast cancer in a male with a polymorphic allele in exon 2 is 2·476 (95% C.I. 1·041 to 5·890).

5.5 Cross-Correlation of Results

A table of the results for all of the male breast cancer cases is shown in Appendix 7.4. This shows the different alleles detected in each of the genes under study.

It is possible to cross-correlate these results in order to see if there are any combinations of alleles of different genes that could be regarded as conferring an increased risk.

The expected frequency of a combination of two of the polymorphisms can be calculated simply by multiplying the observed frequencies of each of the individual polymorphisms together. This can then be compared to the observed frequency of the combination of polymorphisms.

For example, 17/63 cases carry the oestrogen receptor gene exon 2 polymorphism. 38/61 cases carry the BRCA2 exon 2 polymorphism. Both alleles were successfully genotyped in 60 cases. The expected frequency of carriage of both polymorphisms would be $17/63 \times 38/61 = 0.1681$ or 10/60 cases. The observed frequency is 9/60 cases, which is marginally lower than the expected frequency. The carriage of polymorphisms in oestrogen receptor gene exon 2 together with BRCA2 exon 2 therefore does not appear to be a 'high-risk' combination.

There are many more different alleles of the androgen receptor gene and CYP19 gene, therefore this type of analysis for these genes in combination with the other genes as above is more difficult. For this reason, the alleles of exon 1 of the androgen receptor gene containing between 27 and 30 repeats are taken together, and studied in combination with the other genes. In our study, none of the alleles of the CYP19 emerged as a 'high risk' allele. One previous study concluded that the (TTTA)₇ allele was associated with increased risk of female breast cancer (Siegelmann-

Danieli and Buetow, 1999). Another study found the (TTTA)₁₂ allele significantly more frequently in female breast cancer patients than in controls (Kristensen et al, 1998). For this reason, only these two alleles of the CYP19 gene have been included in the cross-correlation of our results.

The cross-correlation of results, showing the observed (Obs) and expected (Exp) frequencies of combinations of alleles is in Table 5-10.

There are clearly no significant differences between the observed and expected frequencies of any of the combinations of alleles studied.

The expected number of cases carrying a BRCA2 mutation and the (TTTA)₇ allele of CYP19 would be three. The observed number was five. Interestingly, two of the five cases were aged only 32 and 39 at diagnosis, and another two of the cases had two and three affected first-degree female relatives respectively.

Five cases were found to have an androgen receptor gene exon 1 containing between 27 and 30 CAG repeats together with a BRCA2 exon2 polymorphism. The expected frequency of this combination would be 3/53 cases. It is therefore possible that carriage of the BRCA2 exon 2 polymorphism, in combination with an androgen receptor gene exon 1 containing greater than 27 CAG repeats confers a particularly increased susceptibility to male breast cancer. However the numbers are too small to allow any meaningful statistical analysis.

Table 5-10 Cross-correlation of the results for all of the genes studied

	CYP17	CYP19 (7 repeats)	CYP19 (12 repeats)	Oestrogen Receptor Gene Intron 1	Oestrogen Receptor Gene Exon 2	BRCA2 Exon 2	BRCA2 Mutations
Androgen Receptor Gene (27-30 repeats)	Exp 5/56 x 47/64 = 0.06557 ≡ 4/56	5/56 x 18/54 = 0.02976 ≡ 2/52	5/56 x 4/54 = 0.006614 ≡ 0/52	5/56 x 49/63 = 0.06944 ≡ 4/55	5/56 x 17/63 = 0.02409 ≡ 1/55	5/56 x 38/61 = 0.05562 ≡ 3/53	5/56 x 12/64 = 0.01674 ≡ 1/56
	Obs 4/56	3/52	0/52	2/55	1/55	5/53	1/56
CYP17	Exp	47/64 x 18/54 = 0.2448 ≡ 13/54	47/64 x 4/54 = 0.05440 ≡ 3/54	47/64 x 49/63 = 0.5712 ≡ 36/63	47/64 x 17/63 = 0.1982 ≡ 12/63	47/64 x 38/61 = 0.4575 ≡ 28/61	47/64 x 12/64 = 0.1377 ≡ 9/64
	Obs	11/54	3/54	37/63	11/63	30/61	9/64
CYP19 (TTTA) ⁷	Exp			18/54 x 49/63 = 0.2593 ≡ 14/54	18/54 x 17/63 = 0.08995 ≡ 5/54	18/54 x 38/61 = 0.2077 ≡ 11/51	18/54 x 12/64 = 0.0625 ≡ 3/54
	Obs			13/54	5/54	12/51	5/54
CYP19 (TTTA) ¹²	Exp			4/54 x 49/63 = 0.05761 ≡ 3/54	4/54 x 17/63 = 0.01999 ≡ 1/54	4/54 x 38/61 = 0.04614 ≡ 2/51	4/54 x 12/64 = 0.01389 ≡ 1/54
	Obs			4/54	0/54	3/51	1/54
Oestrogen Receptor Gene Intron 1	Exp				49/63 x 17/63 = 0.2099 ≡ 13/63	49/63 x 38/61 = 0.4845 ≡ 29/60	49/63 x 12/64 = 0.1458 ≡ 9/63
	Obs				15/63	29/60	9/63
Oestrogen Receptor Gene Exon 2	Exp					17/63 x 38/61 = 0.1681 ≡ 10/60	17/63 x 12/64 = 0.05059 ≡ 3/63
	Obs					9/60	1/63

Chapter 6 - Discussion

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6.1 Androgen Receptor Gene

In this part of the study looking at the androgen receptor gene, we did not observe any overall difference between the median CAG repeat length comparing male breast cancer patients with controls. However, no males in the control group had alleles containing more than 28 CAG repeats, whereas two of the male breast cancer patients had alleles with 29 and 30 repeats respectively. Only one of the male breast cancer patients had an allele containing 18 repeats or less, compared with six of the controls.

To our knowledge, the length of this CAG repeat has been studied in only one group of male breast cancer patients previously (Haraldsson et al, 1998). There was found to be no significant difference between male breast cancer cases and controls.

However, sequences of 30 repeats or more were found only among cases. Our results are consistent with these findings. In addition, it has been observed that women who are carriers of BRCA1 mutations are at a significantly increased risk of breast cancer if they carry at least one androgen receptor gene allele with 28 or more CAG repeats (Rebbeck et al, 1999). We believe that a relatively long CAG repeat sequence within the androgen receptor gene may be implicated in a few cases of male breast cancer. Conversely, a short CAG repeat sequence might offer a degree of protection against male breast cancer.

It is well recognised that Klinefelter's syndrome is associated with an increased risk of male breast cancer (Jackson et al, 1965). One of the male breast cancer patients in our study was known to have had Klinefelter's syndrome. Our study of the androgen receptor gene has enabled us to identify a further two patients who almost certainly had Klinefelter's syndrome.

6.2 BRCA2

Previous Studies

Thirteen previous publications record the frequency of BRCA2 mutations in male breast cancer populations. Four of these were small series (less than 20 cases). Csokay et al (1999) detected mutations in six out of 18 cases. None had a family history of breast cancer. Diez et al (2000) found mutations in three out of 17 cases. However six of the 'cases' were affected females who had an affected first-degree male relative. Wolpert et al (2000) described mutations in two out of a very small series of 14 cases (unselected for family history). Pages et al (2001) reported an even smaller series of 12 affected males who were all from families with at least two cases of female breast cancer. Three truncating mutations and one missense variant were detected. However the missense variant was found in a family containing two cases of male breast cancer.

Four of the series reported larger numbers of cases, but detected relatively few mutations. Friedman et al (1997) found mutations in only two out of 54 cases studied. Mavraki et al (1997) detected mutations in 3 out of 28 cases. However two of the 28 'cases' were affected female relatives of male cases and one of the 'mutations' was a missense variant of unknown significance. Kwiatkowska et al (2001) reported mutations in four out of 37 cases. There were another three cases with missense variants. Ottini et al (2003) reported a series of 25 male breast cancer cases recruited regardless of family history, three of whom were found to carry mutations.

Three moderate-sized series have detected larger numbers of mutations. Couch et al (1996) identified mutations in seven out of 50 cases. The authors stated that the

males were not selected on the basis of family history, however six of the seven cases found to carry mutations had a family history of breast cancer. The highest frequency of BRCA2 mutations reported to date has been in a series of male breast cancer cases from Iceland. Thorlacius et al (1998) found a single founder mutation in 13 out of 34 cases. Another series in which a high frequency of BRCA2 mutations was detected was from Sweden. Haraldsson et al (1998) reported mutations in seven out of 34 cases. Of note was the fact that only one of these cases had a family history of breast cancer.

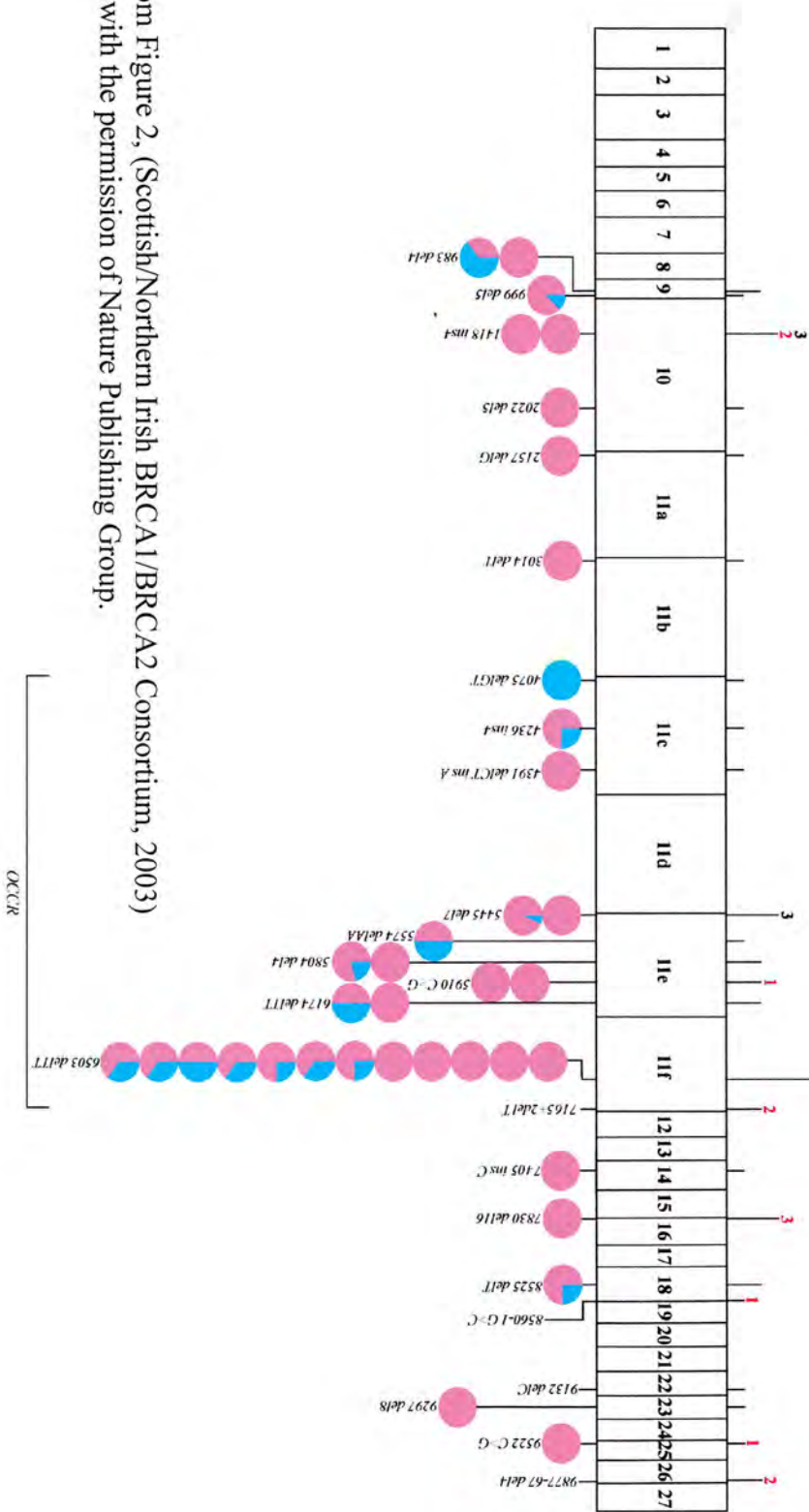
The largest published series reported 124 male breast cancer cases from Israel (Struwing et al, 1999). A common Ashkenazi Jewish founder mutation (6174 delT) was detected in 15 of the cases. However, the study was limited in that only this single mutation was screened for.

Basham et al (2002) published a large study of 94 cases from England. Only five cases were found to carry truncating mutations and only one of these five reported a history of breast cancer in a first-degree relative.

A representation of all BRCA2 mutations found to date in Scotland and Northern Ireland is shown in Figure 6.1. The numbers of mutations found in male breast cancer cases and their location within the BRCA2 gene are highlighted. It can be seen that the distribution of mutations found in males is different from females. Only one of the mutations found in male cases is within the so-called ovarian cancer cluster region (OCCR) that was proposed by Gayther et al (1997). This is consistent with the low number of ovarian cancers found among relatives of the male BRCA2 mutation carriers in this study.

Figure 6.1

Diagrammatic representation of BRCA2 with mutations found in breast cancer cases from Scotland and Northern Ireland. Heights of the vertical lines above the diagram represent the numbers of mutations detected. Actual numbers are given for more frequent mutations and numbers found in male breast cancer cases are highlighted in red. The pink segment of each circle represents the proportion of breast cancers. The blue segment represents the proportion of ovarian cancers.



Modified from Figure 2, (Scottish/Northern Irish BRCA1/BRCA2 Consortium, 2003)
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The BRCA2 mutations that we have identified are discussed in more detail below.

Frameshift Mutations

The 1418 insTTAG exon 10 mutation has previously been reported in a Scottish family containing five cases of female breast cancer (Gayther et al, 1997). This mutation within codon 473 results in a frameshift, which is predicted to produce premature termination of translation at codon 497. However, using PTT, we were not able to demonstrate a truncated protein product. The reason for this is unknown.

The 7830 del16 exon 16 mutation has been reported previously in a Japanese family with four cases of female breast cancer (Katagiri et al, 1998). This mutation also results in a frameshift. The amino acid sequence would be altered such that a termination codon is not produced until further downstream than normal. Exon 16 normally codes for a sequence of 68 amino acids. This mutation is predicted to result in the translation of an abnormal sequence of 90 amino acids. Due to the relatively small size of exon 16 and the close proximity of the mutation the intron / exon border, the methods used in our study for PTT were not suitable to confirm the translation of a truncated protein.

Nonsense Mutations

The 5910 C→G mutation has been reported recently (Risch et al, 2001). We have demonstrated that this mutation in exon 11 results in a truncated protein (Figure 5·6). This is easily explained as the mutation is predicted to change codon 1970 to a termination codon.

The 9522 C→G mutation in exon 25 has been recorded on the Breast Cancer Information Core database. This mutation results in codon 3174 becoming a termination codon and is therefore predicted to result in a truncated protein. Due to the relatively small size of exon 25, the methods used in our study for PTT were not suitable to confirm the translation of a truncated protein.

Splicing Mutations

The process of RNA transcription involves a splicing process whereby the intronic RNA segments are removed, and the exonic RNA segments are joined end-to-end. This process depends upon the identification of certain DNA sequences that are present at the so-called splice junctions (the boundaries between introns and exons). The DNA sequences that are present at the exon/intron and intron/exon boundaries (otherwise known as splice junctions) are shown in Figure 6·2. The nucleotides highlighted in bold are invariant and are vitally important for normal splicing to occur. There is evidence from other genes (e.g. p53) that base changes within a short distance of these splice junctions may also affect the splicing process (Varley et al, 2001).

The branch site is another sequence within the intron that is also important for splicing. This is also shown in Figure 6·2 and it is usually located anywhere from 6 to 59 nucleotides upstream of the splice acceptor site (Maquat, 1996).

The RNA splicing mechanism involving the branch site is illustrated in Figure 6·3. The exonic RNA segments are spliced together and the intronic RNA is released in the form of a lariat structure.

Figure 6.2

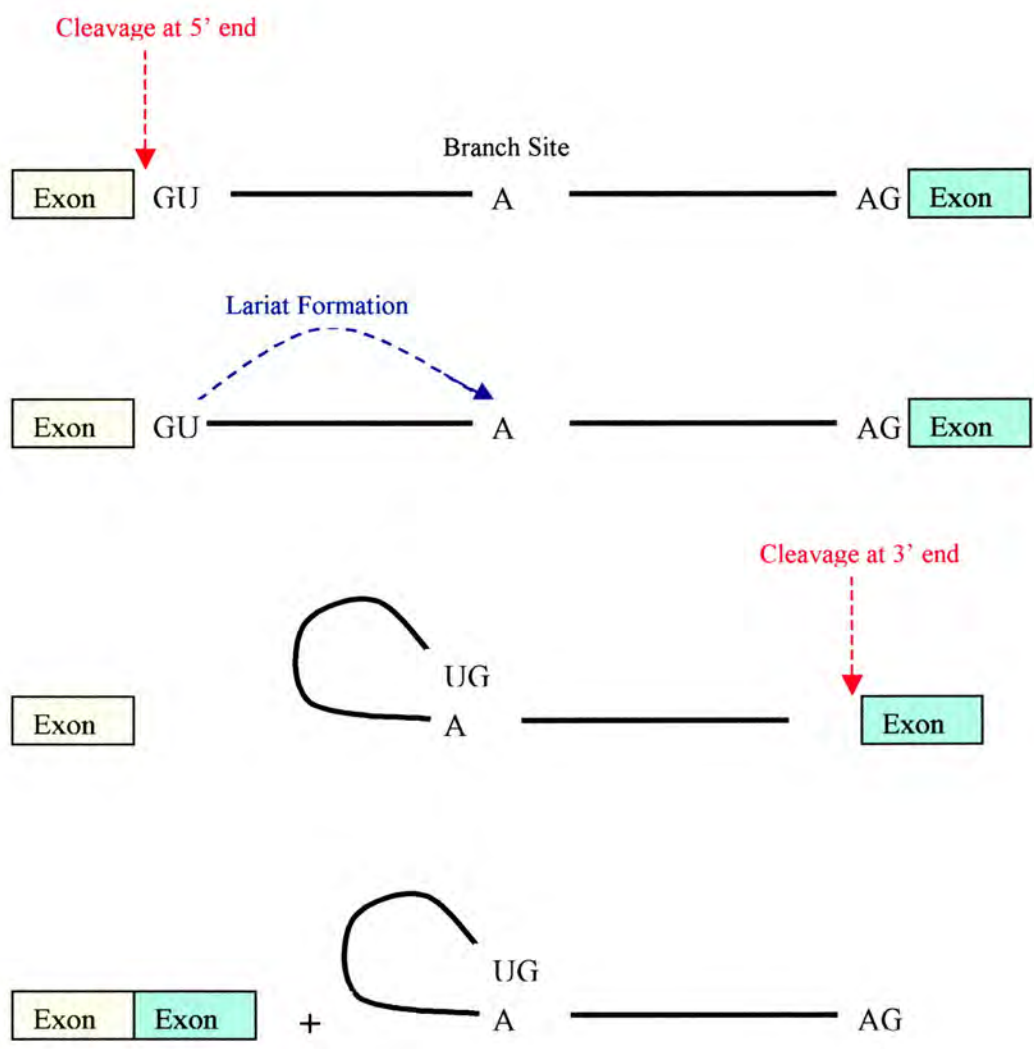
Consensus sequences at the splicing sites within introns



Modified from Figure 1.15, p18 (Strachan & Read, 1997)

Figure 6.3

The mechanism of RNA splicing



Modified from Figure 1.16, p18 (Strachan & Read, 1997)

The BRCA2 mutations found in our study that are thought to affect splicing are now discussed below.

The 7165+2 delT mutation has not been previously recorded. It will alter the sequence of the splice donor site at the 5' end of intron 12.

5'-CCAGATGgttaaaatt-3' Wild-type

5'-CCAGATGgttaaatt-3' Mutant

Whilst the invariant GT within the splice donor site is unaffected, the third base is changed T→A. The effect of this is unknown. A mutation within the splice donor site of BRCA2 intron 7 (859+2 T→G) has been shown to result in the deletion of exon 7 (Pyne et al, 2000). It is possible that the intron 12 mutation detected in our study might have a similar effect.

The 8560-1 G→T mutation has not previously been recorded. It will alter the sequence of the splice acceptor site at the 3' end of intron 18.

5'-aattgtccagATTTCTGCTAACAGTACTC-3' Wild-type

5'-aattgtccatATTTCTGCTAACAGTACTC-3' Mutant

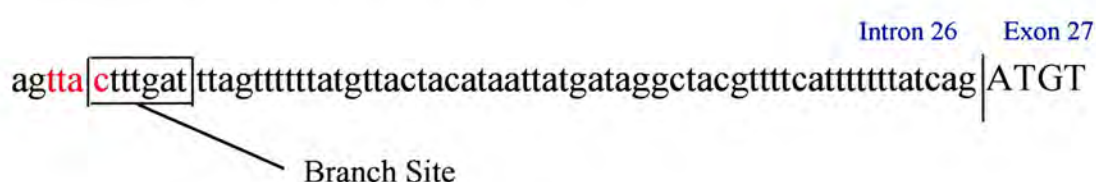
The invariant AG is altered and it is therefore highly likely that splicing will be affected.

The 9877-67 delTTAC variant in intron 26 has been reported in two unrelated cases with ovarian carcinoma (Takahashi et al, 1996). The authors suggested that this deletion may affect splicing, but did not explain how.

Taking the consensus sequence of the intronic branch site (Figure 6·2), the closest point to the intron/exon border where this sequence is found in BRCA2 intron 26 is shown in Figure 6·4.

Figure 6·4

The sequence of the 3'-end of BRCA2 intron 26



It can be seen that deletion of the TTAC, as shown in red, would result in a C→G change in the first base of the branch site sequence. In vitro studies have shown that mutation of the invariant adenine within the branch site has the most significant effect upon splicing, but change in the first base from C→T results in a smaller effect (Reed & Maniatis, 1988). However, the effect of a C→G change in the first base of the branch site sequence is not known. An effect on splicing is likely to be important, though, because there is recent evidence suggesting that exon 27 is involved in the interaction between BRCA2 and RAD51 (Donoho et al, 2003). If this variant does have an effect on splicing then it may alter the level of expression of the BRCA2 gene, or it may result in an abnormal protein equivalent to a deletion of exon 27. Neither of these possible effects would result in an abnormal protein that would be visible on PTT of exon 27. This is in agreement with our results.

We have identified germline BRCA2 mutations in 19% of male breast cancer cases studied from the South East Scotland region. This is a higher frequency than previously reported from the U.K. (Mavraki et al, 1997; Basham et al, 2002) or the U.S.A. (Couch et al, 1996; Friedman et al, 1997). It is possible that germline BRCA2 mutations may account for a higher proportion of male breast cancer cases than previously thought. Previous studies may have missed potentially important mutations within splicing regions or may have classified such mutations as unimportant. A number of previous studies have shown that BRCA2 mutations in male breast cancer are not necessarily associated with a positive family history of breast cancer, the proportion of cases carrying BRCA2 mutations that have a positive family history ranges from 0% to 86% (Csokay et al, 1999; Couch et al, 1996). Our study is in agreement with this, although positive family history was recorded for six of our twelve cases. Analysis of the family pedigrees identifies four further males in whom no BRCA2 mutation was detected, but whose family history is suggestive of there being a genetic abnormality within the family. It is possible that our screening of the BRCA2 gene has missed a number of mutations (see below). Alternatively, these males may carry a mutation in another gene, for example BRCA1.

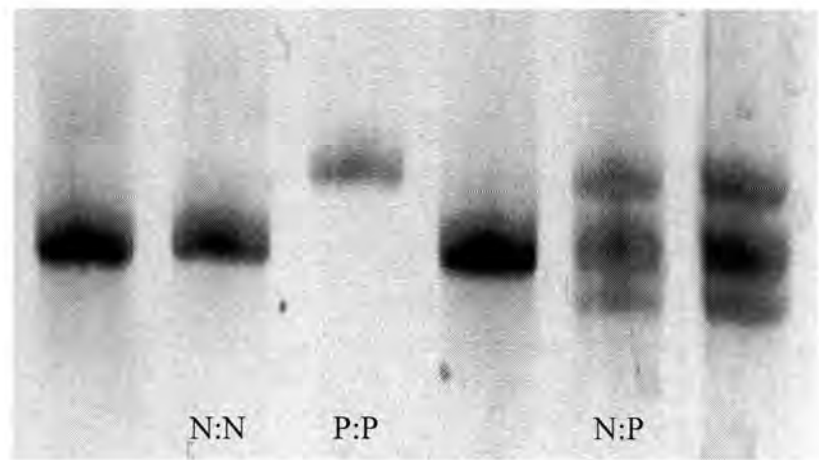
Previous studies have found the frequency of the 203 G→A exon 2 polymorphism to be between 17% and 22% in control populations (Couch et al, 1996; Friedman et al, 1997). These studies did not find a significantly higher frequency of the polymorphism among male breast cancer patients. It would appear that this polymorphism is commoner in the S.E. Scotland population as a whole (45% in our study). We have also shown that a significantly higher proportion of the male breast

cancer cases in our study (62%) carry the polymorphism. There are several possible reasons for this. This polymorphism may be in linkage disequilibrium with the exon 16 mutation, which is the commonest mutation found in this male breast cancer population. It is also possible that this polymorphism may in some way alter the expression of the BRCA2 gene, as has been suggested (Mariani-Costantini et al, personal communication).

A small number of samples studied (four cases and five controls) were found to be homozygous for the BRCA2 exon 2 polymorphism. These were detected on heteroduplex analysis as a bandshift (Figure 6.5). One can only speculate on how a single base change present on both alleles might alter the conformation of the DNA sufficiently to slow its migration through the non-denaturing gel.

Figure 6.5

Heteroduplex analysis showing different alleles of BRCA2 exon 2



N:N = Homozygous wild-type N:P = Heterozygous for polymorphism
P:P = Homozygous for polymorphism

Another curious finding is that some of the sequencing films show apparent homozygosity for mutations (Figures 5·9 and 5·10). This could be explained if the majority of the DNA in that particular sample was derived from tumour tissue, and the wild-type allele has been 'knocked out'. However, all three of the cases carrying the 7830 del16 exon 16 mutation (Figure 5·10) were heterozygous for the 203 G→A exon 2 polymorphism, therefore the DNA from each of these samples must contain two alleles of BRCA2 exon 2. It is therefore possible that only part of the wild-type BRCA2 allele has been 'knocked out'. Another possibility may be that the wild-type allele has not been amplified during PCR, perhaps due to the presence of a polymorphism that inhibits primer annealing.

All of the other polymorphisms identified in our study have also been described previously (Tavtigian et al, 1996; Takahashi et al, 1996; Friedman et al, 1997; Breast Cancer Information Core).

All variant bands found on HA correlated with visible sequence variants on subsequent cycle sequencing. We can therefore assume that the specificity of HA and the sensitivity of sequencing in our study were both 100%. According to published series, the sensitivity of HA appears to be between 60% and 100%. To evaluate the sensitivity of HA in this study would have required sequencing all PCR products, which clearly was not practical. As already indicated, for the screening of BRCA2, approximately 85% of PCR's were ultimately successful. Assuming a sensitivity for HA of 80%, then we can estimate that in this study the sensitivity for the screening process of BRCA2 as a whole was 0.85×0.80 , or 68%. It is therefore possible that we have missed almost one third of the BRCA2 mutations present in these cases.

A review of studies reporting survival in female breast cancer occurring in association with BRCA2 mutations, stated that no conclusion could be drawn as to whether carriage of a BRCA2 mutation was associated with a worse or better prognosis (Verhoog et al, 2000). However, there are no previously published data on outcome in male breast cancer associated with BRCA2 mutations. Our data indicate that carriage of a germline BRCA2 mutation in a male who has developed breast cancer has no significant effect on age at diagnosis, histological classification of the tumour, disease stage at presentation or risk of loco-regional relapse within 5 years, or survival.

Other studies have reported associations between BRCA2 mutations and a variety of other cancers. However, in our study, within the group of families in whom BRCA2 mutations have been detected, there do not appear to be an excess of other cancers, although our analysis has largely been confined to first-degree relatives.

Use of the Scottish public records by an experienced genealogist has provided us with detailed and complete family pedigrees in the majority of cases. Information provided by the Cancer Registry has also given us accurate data on cases of cancer among relatives of the male probands. The age of onset of cancer is known for the majority of affected relatives, rather than simply the age at which the individual died. This allows a more accurate estimate of risk.

We have shown that the risk of developing breast cancer among female first-degree relatives of male breast cancer probands is significantly increased if the male is a carrier of a BRCA2 mutation. However, if the male does not carry a BRCA2 mutation, then the risk of developing breast cancer in his first-degree female relatives

is no greater than that of the general population. This suggests that BRCA2 mutations account for the majority of cases of familial breast cancer, when there is an affected male within the family. It would also appear that intensive screening of female relatives of male breast cancer cases is only necessary if a BRCA2 mutation is found in the male proband.

Previous population-based studies have estimated the breast cancer penetrance, by age 70, for BRCA2 mutations to be 26% (Satagopan et al, 2001), 28% (Warner et al, 1999), 37% (Thorlacius et al, 1998) and 56% (Anglian Breast Cancer Study Group, 2000). For BRCA1/2 mutations combined, penetrance by age 70 has been estimated to be between 40% (Hopper et al, 1999) and 56% (Struewing et al, 1997). Other studies have given higher estimates for BRCA2 mutations of 79.5% (Easton et al, 1997) and 84% (Ford et al, 1998). However, these studies were in 'high-risk' breast cancer families, each containing multiple cases of female breast cancer.

We have estimated that the breast cancer penetrance for the BRCA2 mutations, as a whole, in our study to be about 62% for females by the age of 72-73. The male breast cancer cases in our study were not selected on the basis of family history. The potential for ascertainment bias is therefore eliminated. However, the estimate of 62% in our study is higher than the estimates of penetrance given in the previously published population-based series shown above. It is possible that BRCA2 mutations associated with male breast cancer carry a higher risk for females (i.e. these mutations have a higher penetrance). Alternatively, families that contain affected males may also carry other genetic variants that either increase breast cancer risk themselves or modify the penetrance of BRCA2 mutations in females. It is known from the studies of the Icelandic founder BRCA2 mutation, that the penetrance of a

single BRCA2 mutation can vary between different families (Thorlacius et al, 1997). This provides further evidence for the existence of other factors that modify the effects of BRCA2 mutations.

One way of attempting to identify these factors is by looking again at BRCA2 function. It has been shown that chromosomal instability in cells without functional BRCA2 normally results in death of these cells due to checkpoint mechanisms and apoptosis (Patel et al, 1998). Inactivation of BRCA2 alone therefore cannot be sufficient to result in carcinogenesis. It is possible that secondary mutations occur that either inactivate the cell-cycle checkpoints or prevent apoptosis. Cells containing damaged DNA could then remain viable and have the opportunity to become tumour cells.

The p53 gene mediates cell-cycle arrest and apoptosis after DNA damage has occurred (Levine, 1997). There is evidence that p53 interacts functionally with BRCA2 and RAD51 *in vivo* (Marmorstein et al, 1998). It has been shown in a murine model that loss of functional BRCA2 together with p53 frequently leads to the development of breast tumours (Jonkers et al, 2001). Another study has suggested that the loss of BRCA2 alone allows chromosomal abnormalities to accumulate in cells until eventually, p53-mediated apoptosis occurs. When BRCA2 and p53 are both lost, then DNA damage cannot result in either cell-cycle arrest or apoptosis (Cheung et al, 2002). Mutations in the p53 and Bub1 genes inactivate cell-cycle checkpoints, resulting in transformation of cells homozygous for truncated BRCA2 into tumour cells (Lee et al, 1999). Amplifications of the AURORA-A gene and associated increased protein expression occur in 12% - 62% of epithelial carcinomas.

This has been suggested as an alternative mechanism for disruption of the normal cell-cycle checkpoint (Anand et al, 2003).

Knowledge of the process of homologous DNA repair is now extending beyond the interaction between BRCA2 and RAD51. Fanconi anaemia is an autosomal recessive disorder associated with an increased risk of cancer. Interactions between Fanconi anaemia proteins and BRCA2 have been demonstrated, although how these proteins contribute to DNA repair is not fully understood (Hussain et al, 2003). The protein kinases ATM (which is mutated in ataxia-telangiectasia) and CHEK2 are also involved in the mechanism by which cells are able to recognise double-strand DNA breaks (Venkitaraman, 2003). Exactly how they interact with the other components of the DNA repair pathway is not yet known.

Further understanding of the mechanisms by which BRCA2 is involved in DNA repair, in particular its interaction with the other genes mentioned above, will inevitably lead to better identification of individuals at increased risk of breast cancer. It may also provide new opportunities for cancer treatment.

6.3 CYP17

This is the first study that attempts to evaluate the possible role of a polymorphism of the CYP17 gene in the development of male breast cancer.

We have found that the polymorphic T→C substitution within the CYP17 gene occurs significantly more frequently in male breast cancer patients than in male controls. These results are consistent with other reports that there may be a hormonal contribution to the development of male breast cancer (Jackson et al 1965, Lobaccaro et al 1993, Wooster et al 1992).

We have also studied a small group of female breast cancer patients from the same geographical region. We found no association between the presence of a C allele of the CYP17 gene and female breast cancer. The first study that looked at this polymorphism in female breast cancer patients (Feigelson et al 1997), found a significant association only after sub-selecting those patients that presented with advanced disease. The second, and much larger, study (Dunning et al 1998) did find a small increase in the risk of breast cancer associated with the C allele, but this increase was not statistically significant. It is possible that the T→C polymorphism in the CYP17 gene is associated with higher levels of serum oestrogens, but because the endogenous oestrogen level is higher in females than in males, a further increase does not have a significant effect. The association between an increase in the levels of serum oestrogens and the development of breast cancer may therefore be detectable only in males, or perhaps as previously suggested (Dunning et al 1998), in post-menopausal females.

Several recently published studies looking at the CYP17 polymorphism in prostate cancer, have produced contradictory results. One study found the reverse of the

association that we detected in male breast cancer. Men homozygous for the T (wild-type) allele had a significantly higher risk of prostate cancer than those homozygous for the C (polymorphic) allele (Habuchi et al, 2000). However, another study found an increased risk of prostate cancer in men homozygous for the polymorphic allele (Gsur et al, 2000).

Further studies of this type are required, specifically to test for any association between the polymorphism of the CYP17 gene and levels of serum oestrogens in males.

6.4 CYP19

This is the first study that attempts to determine whether the length of the tetranucleotide repeat sequence within intron 5 of the CYP19 gene influences the development of male breast cancer. Two recent studies have determined the distribution of alleles among female breast cancer patients and controls. The first of these studies (Kristensen et al, 1998) found five different alleles containing 7,8,9,11 and 12 repeats. The allele containing 12 repeats was found significantly more frequently in female breast cancer patients than in controls. The second of the studies (Siegelmann-Danieli and Buetow, 1999) described eight different alleles by PCR product length. Details of the numbers of tetranucleotide repeats were not given. Alleles of 168bp and 171bp in length were found, presumably corresponding to the two different alleles containing 7 repeats found in our study, although the 3-bp deletion was not characterised. However, this deletion has been described previously in a Japanese study (Kurosaki et al, 1997). From their study, Siegelmann-Danieli and Buetow (1999) conclude that the 171bp allele represents a high-risk allele, whereas

the 187bp and 191bp alleles (corresponding to 11 and 12 repeats respectively, from our data) are considered to confer low risk. These conclusions are contradictory to those drawn by Kristensen et al (1998).

Contrary to published (and mutually incompatible) findings in female breast cancer, our study found no significant differences in distribution of alleles between male breast cancer cases and controls.

Patient MBC8, who has an allele with 13 repeats, was diagnosed with breast cancer at a very young age (26 years), but has no family history of cancer. This allele was inherited from his father and therefore does not represent a de-novo mutation. It certainly represents a rare variant within the South East Scotland population, but is most likely to be an incidental finding.

6.5 Oestrogen Receptor Gene

In this study we examined the frequencies of two oestrogen receptor gene polymorphisms in a group of male breast cancer patients from the South East of Scotland. We have demonstrated an association between a polymorphism in exon 2 of the oestrogen receptor gene and male breast cancer. There was no such association with a polymorphism in intron 1. It is possible that the exon 2 polymorphism has an effect on the level of transcription of the gene, and this would be an interesting area for further study. A possibility for further study would be to perform immunohistochemistry to measure the oestrogen receptor status of the primary tumours. It would then be possible to determine whether there are any associations between polymorphisms in the oestrogen receptor gene and oestrogen receptor expression.

This is the second modifying polymorphism that we have associated with an increased risk of male breast cancer. We believe that this further illustrates the potential importance of these low-penetrance genes in the development of breast cancer. Larger studies are required to determine whether the associations between the modifying polymorphisms and male breast cancer are strengthened by the addition of more male breast cancer cases.

6.6 Cross-Correlation Of Results

The expected number of cases carrying a BRCA2 mutation and the (TTTA)₇ allele of CYP19 would be three. The observed number was five. Interestingly, two of the five cases were aged only 32 and 39 at diagnosis, and another two of the cases had two and three affected first-degree female relatives respectively. The numbers of cases are very small, but it is possible that the presence of this allele of CYP19 has a modifying effect upon the penetrance of some of the BRCA2 mutations found in our study.

Five cases were found to have an androgen receptor gene exon 1 containing between 27 and 30 CAG repeats together with a BRCA2 exon2 polymorphism. The expected frequency of this combination would be 3/53 cases. It is therefore possible that carriage of the BRCA2 exon 2 polymorphism, in combination with an allele of exon 1 of the androgen receptor gene containing greater than 27 CAG repeats, confers a particularly increased susceptibility to male breast cancer. However the numbers are again too small to allow any meaningful statistical analyses.

6.7 Conclusions

Our study of 64 male breast cancer cases from the South East of Scotland is one of the largest and most comprehensive reported to date. We performed comprehensive screening of the BRCA2 gene and detected mutations in 19% of cases. This is an appreciably higher proportion than previously recorded for U.K. populations. The explanation for this may lie in genetic differences between the Scottish and other U.K. sub-populations and/or in our recognition of pathogenic splice-site mutations previously overlooked. BRCA2 mutations in male breast cancer are not necessarily associated with a positive family history of breast cancer, although half of our series did have affected relatives. In four additional cases, a positive family history was found despite absence of a demonstrable BRCA2 mutation, implying either that some BRCA2 mutations have been missed or that some cases may be attributable to mutations in other genes (such as BRCA1) not examined comprehensively in this study.

We have also shown that a significantly higher proportion of the male breast cancer cases in our study carry the 203 G→A BRCA2 exon 2 polymorphism compared to a control population. The effects of this polymorphism, particularly upon gene expression, require further evaluation.

Carriage of a germline BRCA2 mutation in a male who has developed breast cancer appears to have no significant effect on age at diagnosis, histological classification of the tumour, disease stage at presentation, risk of loco-regional relapse within 5 years, or survival.

The risk of developing breast cancer among female first-degree relatives of male breast cancer probands is significantly increased, only if the male is a carrier of a BRCA2 mutation.

Breast cancer penetrance to age 73, of germline BRCA2 mutations in females within our population is calculated as around 62%.

The findings presented in this study also indicate that the CAG repeat sequence within the androgen receptor gene may, in some cases, be one useful molecular marker to identify males at increased risk of developing breast cancer. Larger studies are required to further define the importance of this CAG repeat in male breast cancer.

We have found that polymorphisms in the CYP17 and estrogen receptor genes occur significantly more frequently in male breast cancer patients than in male controls.

We have not found any association between a polymorphic tetranucleotide repeat sequence in the CYP19 gene and male breast cancer. It is possible that the (TTTA)₇ allele of CYP19 has a modifying effect upon the penetrance of some of the BRCA2 mutations found in this study.

Subsequent to our study, an association between a variant in the CHEK2 gene and male breast cancer has been published. This is another interesting area that requires further evaluation.

Chapter 7 - Appendix

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7.1 Family History Form

Male Breast Cancer Study

Name

.....

Date of Birth

...../...../.....

G.P.'s Name

.....

Surgery Address

.....
.....
.....

Place of Birth

.....
.....

Children (Names)	Date & Place of Birth	Present Address or Date & Place of Death	Health
Brothers / Sisters (Names)			

Male Breast Cancer Study

Father's Family

Names	Date & Place of Birth	Address or Date & Place of Death	Health
Father			
Paternal Grand Father			
Paternal Grand Mother (Maiden Name)			
Paternal Uncles/Aunts/Cousins			

Male Breast Cancer Study

Mother's Family

Names	Date & Place of Birth	Address <u>or</u> Date & Place of Death	Health
Mother (Maiden Name)			
Maternal Grand Father			
Maternal Grand Mother (Maiden Name)			
Maternal Uncles/Aunts/Cousins			

7.2 BRCA2 Primers

Exon	Primer Sequence Forward / Sense	Reverse / Anti-sense	Size (bp)	T _a (°C)
2	CTCAGTCCACATAATAAGGAAT	ACACTGTGACCGTACTGGGTTT	256	52-47
3	TCTGGGTCACAAATTGTCTGTCA	TTCCTAGTTTGTAAGTTCCTCCAGTC	356	52-47
4	AGAATGCAAATTATATAATCCAGAGTA	AATCAGATTCACTTATATAGAACAATA	249	52-47
5	AACAATTATATGAATGAGAATC	AATTGTTAAGTTTATTTTATTA	220	55-45
6	CCACAAAGAGATAAGTCAGGTA	TGTAAATCTCAGGGCAAAAGGTA	234	52-47
7	TAAGTGAATAAAGAGTGAA	AACAGAAAGTATTAGAGATGAC	275	52-47
8	AATAGTAGATGTGCTTTTGA	ACATATAGGACCAGGTTTAGAGAC	285	52-47
9	CTAGTGATTTAAACTATTAATTTTG	GTTCAACTAAACAGAGGACT	164	50
10A	TATAAATATTAATGTGCTTCTGT	AAAGGCTTCTGATTTGCTAC	374	50
10B	ATCTGAAGTGGAAACCAAAATGATAC	ACGTGGCAAGAATTTCTCTGAAGTAA	270	50-47
10C	TTTCAGAAAAAGACCTATTAGACA	CTTTTGTATACCCTGAAATGAAGAAG	260	50
10D	TAAAGCAGGCAATATCTGGAACCTCT	GTGGATATTAACCTGCATTCTTCAA	280	52-47
10E	TATGTCCAAATTTAATTGATAAT	AAACACAGAAAGGAATCGTCATC	320	55-45
11A	GATGGTACTTTAATTTTGTCACTTIG	TTCCTTATTAATTTTGTCTTCTTAT	220	52-47
11B	CTAGCTCTTTTGGGACAATTTCTGAGG	TTTCATGATCATATATAAAGAATT	300	55-45
11C	CAAAAGTGAATACAGTGATAC	ATAATTTTCATTTAAAGCACATACAT	250	52-47
11D	TCTAGAGGCAAGAATCATATA	TCAATGTCTGAGAAAAAGTTC	305	50-45
11E	TTCAAAAAATAACTGTCAATCC	GTTGCTTGTATATCACCCTGT	230	50
11F	AACCCATTTTCAAGAACTCTACCA	CTGAAGCTACCTCCAAAACCTGTG	274	50
11G	ACAAATGGGCAGGACTCTTAGG	CCTGCTTGAAAAATAACATCTG	300	50

Exon	Primer Sequence Forward / Sense	Reverse / Anti-sense	Size (bp)	T _a (°C)
1IH	AGTTGTTTCTGATTGTAAAAATAGTC	GCTGCTGTCCTACCTGACCA	310	52-47
1II	GATGCTGATCTTCATGTCATAA	TACCTCTGACAGAAGTTTCCTCACTA	300	50
1IJ	GGGTTAGGGGCTTTTATTC	TATCAGTTGGCATTATTAATTTT	237	50
1IK	CTTCAAGTAATGTCAATGATTCTGTC	CTGGCAGCAGTATATTTGTTATCT	202	53
1IL	ATTACTGAAAAATTACAAGAGAAATA	AAAAAGTTAAATCTGACTCAAAATCT	244	60-50
1IM	TTTATGAAGGAGGGGAAACACTCA	TTCAGAAATTAAGGAAAAAGTTATGC	282	50
1IN	AAAAAATATTAGTGTGCGCCAAAGAG	TGTATGAAAAACCCCAACAGAGTAGG	278	53
1IO	GTTAAACACAAAAATACTGAAG	CATTGATGGCTAAAAACTGGTG	233	50
1IP	TCATACAGCTAGCGGGAAGAAAA	TGGCACCCACAGTCTCAATAG	265	52-47
1IQ	CTGCCCCAAAGTGTAAGAAAT	AATGACTGAATAAGGGGACTGAT	230	52-47
1IR	GTCCTGCAACTTGTACAC	GATTTTGTCAITTTTCAGC	230	52-47
1IS	AACCAGAAAGAATAAATACT	TCCTCAACGCAATATCTTCAT	334	50
1IT	TTCCAAAAGTAATATCCAAATGTA	ATTTTGAATTAATCTCGTTGT	291	52-47
1IU	AAGACAATTTACAGACAGT	TGAAGCTTCCCTATACTACAT	300	55-45
1IV	CACCTTGTGATGTAGTTTG	TTGGATATTAATGTTCGAGTA	350	50
1IW	AAAGTAACGAACATTCAGACCA	CTGGGTTTCTCTTATCAACACGA	310	52-47
1IX	AGTCTTCACTATTCACCTACG	GTGAGACTTTGGTTCTAAT	280	55-48
1IY	TTCAACAAGACAAACAACAGT	TGTCAGTTCAATCATCTTCATAAA	270	52-47
1IZ	TTACTCCAAAGATTCAGAAAAACTAC	AGCATACCAAGTCTACTGAATAAAC	290	50
12	AGGTCACTATTGTGTGAAG	AGTGGCTCATGTCTGTAAT	358	55-45
13	TAAAGCCTATAATTGTCTCA	CTTCTTAACGTTAGTGTCAAT	271	55-45

Exon	Primer Sequence Forward / Sense	Reverse / Anti-sense	Size (bp)	T _a (°C)
14A	ATGTAGCAAAATGAGGGTCTG	GTCTGCCCTGTAGTAATCAAGTGT	289	52-47
14B	CTTCAAGCAATTAGCAGTTTCAG	TGCTGCTTGATTGGAGTTGTT	292	55-50
14C	TATTAACTTGAGAGAAAACAGACA	CAAAAGGGGAAAAACCATCAG	250	55-50
15	GGCCAGGGGTGTGCTTTT	AGGATACTAGTTAATGAAATA	314	55-45
16	TTTGGTAAATTCAGTTTGGTTT	AACACACAATCTTTTGCATAGA	330	52-47
17	CAGAGAATAGTTGTAGTTGTTGAA	AGAAACCTTAACCCATACTGC	306	52-47
18A	ATTCAGTTTATTCTCAGTTATTC	ACCACCCATCTGTAAGTTCA	296	60-50
18B	ATCTGAAACTTCTAGCAATAA	TTAACTGAATCAATGACTG	281	60-50
19	AAGTGAATATTTTAAAGGCAGTT	TATATGGTAAAGTTTCAAGAAT	296	60-50
20	CACGTGCGCTGGCCTGATAC	ATGTTAAATTCAAAGTCTCTA	296	60-50
21	GGGTGTTTATGCTTGCTTCT	CATTCAACATATTCCTTCCTG	304	60-50
22	TTTTGTTCTGATTGCTTTTATTC	AATCATTTTGTAGTAAGGTCAT	314	60-50
23	ACTTCTCCATTCGATCTTCTCA	AAAACAAAACA AAAAATTCAACATA	290	55-45
24	CAGTTTGTATAAGTGCTTGT	AGCTCCAACATAATCATAGA	290	60-50
25A	TAAAAATTCATCTAACACATCTAT	ACCTCAACAGTATTTTCATT	302	55-45
25B	AATCCCAAAATCAGGCCCTTCTACTT	ATTTCGCCATTCGCCCATCT	283	60-50
26	AAGGAAATACTTTTGGAACACATAA	TTTACTAGGTATACAAACAGAA	299	50-45
27A	TAGGAGTTAGGGGAGGAGACTGTGT	TTTCTCCCTTTACAAGACTTTGAAG	252	60-50
27B	TTGTATGGCCAAAAGGAAGTCTG	TTTTTAAATGGAGTCACTGAGGAGA	285	60-50
27C	AAGGCATTCAGCCACCCAAGGAGT	CAACGTCGTTTCAGTCTGAGATAATC	272	55-48
27D	AGGAGAAAAACAATTATATCTGTCA	GTGGTTGAAATTATATTCACAGTCTT	264	60-50

7.3 Additional Primers

Name	Mutation / Polymorphism	Sequence (5'→3')	Length (bp)	T _a (°C)
10xF 10xR	1418 ins TTAG	GAAGTGACAAAATCTCCAAG GGTCACATGAAGAAATATGC	126	50
11f2F 11f2R	3199 A→G	GCAACCCCAAGTGTCAATTAA ATCAAGGAGATGTCGATT	123	50
11u2	5910 C→G	GCGTGCTACATTCATCATTA	159	55-50
16x	7830 del 16	GCAATGTTTAGAAACGCCAT	146	52-47
17a2	8034-14 T→C	GAAATAAGCTTTGGATCCAC	120	55-50
19a2	8560-1 G→C	AAGTTTGGTATACCAGCGAG	155	55-50
25a2	9522 C→G & 9485-16 T→C	TATGAGGCTTAATAATGTCC	151	55-50
27a2	9877-67 del TTAC	CTCACAAATTAGGAGAAGACATCTGA	150	55-50
BRCA1-2800-F BRCA1-2800-R	2800 del AA	GAAATGCAGAAAGAGGAATGT CAGGCTTGATATTAGACTCA	172	52-47

Note : Where only one primer is shown, this is the 'reverse' primer and is used in conjunction with the original 'forward' primer

7.4 Table Of Results For Male Breast Cancer Cases

Case	Age	Fem Rels	BRCA2		Cyp17	ARG		CYP19		ESR1	
			Ex2	Mut						Intron1	Exon2
MBC1	66	0	G:G		T:C	20		7 (-3)	8	T:C	G:C
MBC2	69	0	G:A		T:C	22		7 (-3)	12	T:C	G:G
MBC3	40	0	G:A	M	T:C	21		7	11	T:C	G:G
MBC4	51	0	G:G		T:C	25		7 (-3)	11	T:T	G:G
MBC6	68	0	G:A		C:C	15		7 (-3)	7	T:T	G:G
MBC7	64	0	G:G		C:C	22		7 (-3)	7 (-3)	C:C	G:C
MBC8	27	0	G:G		T:T	24		7 (-3)	13	T:C	G:C
MBC9	75	0	G:A		T:C	22		7 (-3)	11	T:C	G:G
MBC10	58	0	G:A		C:C	19		7 (-3)	11	T:C	G:G
MBC11	65	0	G:G		T:T	21		11	11	T:C	G:C
MBC12	86	0	G:A		T:C	20		7	8	T:C	G:C
MBC13	71	0	G:G		T:T	23		7	11	T:C	G:G
MBC14	55	0	G:A		T:C	25		7	11	T:T	G:G
MBC15	40	0	G:A		T:T	23		7 (-3)	12	T:C	G:G
MBC16	75	0	G:A		T:C	29		7 (-3)	11	T:T	G:C
MBC17	67	0	G:G	M	T:C	23	25	8	11	T:C	G:G
MBC18	55	1	G:A	M	T:C	24		7 (-3)	11	T:C	G:G
MBC19	74	0	G:A		T:C	24		7 (-3)	8	T:T	G:G
MBC20	49	0	G:G		T:T	20		8	11	T:T	G:C
MBC22	59	0	G:G		T:C	25		11	11	T:C	G:G
MBC23	73	0	G:A		T:C					T:C	G:C
MBC24	64	0	G:A		T:C	25		7 (-3)	7 (-3)	T:T	G:G
MBC25	82	0	G:G		T:C	22		8	12	T:C	G:G
MBC26	79	0	G:A	M	T:T	22		7 (-3)	11	T:C	G:G
MBC28	56	1	G:G	M	C:C	23		7 (-3)	8	T:T	G:G
MBC29	72	0	G:G	M	T:C			7 (-3)	7	T:C	C:C
MBC30	30	1	G:G		T:T	22		7 (-3)	7 (-3)	T:C	G:C
MBC31	75	2	G:A	M	T:C	27		7	11	C:C	G:G
MBC32	72	0	G:A		T:C	23		7 (-3)	8	C:C	G:G
MBC33	76	0	G:A		T:C	24		7	11	C:C	G:G
MBC34	75	0	G:G		T:C	22		11	11	T:C	G:G
MBC35	59	0	G:A		T:T	30		7 (-3)	7	T:T	G:G

Sample	Age	Fem Rels	BRCA2		Cyp17	ARG		CYP19		ESR1	
			Ex2	Mut						Intron1	Exon2
MBC36	48	0			T:C	24		7	11	T:C	G:G
MBC38	48	0	G:G		T:C	24		7	8	T:C	G:G
MBC40	81	0	G:A		T:C	26		7 (-3)	8	C:C	G:G
MBC42	70	0	G:A		T:C	19	25			T:C	G:G
MBC44	70	0	A:A		T:C					T:C	G:G
MBC46	68	1	G:G		T:C	25				T:C	G:C
MBC49	76	0	G:A		T:C	27		7	7	T:T	G:G
MBC50	54	0	G:A		T:T	22		7	11	T:C	G:C
MBC51	64	0	G:A		T:C					T:C	G:G
MBC53	67	0	G:A		T:C	20				T:C	G:C
MBC54	49	1	G:A		T:C	24	27	7 (-3)	11	T:C	G:G
MBC55	73	1	G:A	M	T:C			7 (-3)	12	T:C	G:G
MBC56	32	0	G:A	M	T:T	22		7	11	T:C	G:G
MBC57	71	0	A:A		T:C	24					
MBC58	73	0	A:A		T:C	21		8	11	C:C	G:G
MBC59	73	0	A:A		T:T	22		7	11	T:C	G:C
MBC60	85	0	G:A		T:C	21		7 (-3)	11	C:C	G:G
MBC61	81	0	G:G	M	T:C					C:C	G:G
MBC62	51	0	G:A		T:C					T:C	G:C
MBC63	66	3	G:G	M	T:C	22		7	11	T:T	G:G
MBC64	66	0			T:T	21		7 (-3)	11	T:T	G:G
MBC65	76	0	G:G		T:T	26		7	7	C:C	G:G
MBC66	82	1			T:T	21		11	11	T:C	G:G
MBC67	82	0	G:G		T:C	20		11	11	T:C	G:G
MBC68	85	0	G:A		T:T	25		7 (-3)	11	C:C	G:G
MBC72	54	0	G:A		T:C	24		7 (-3)	7	T:C	G:C
MBC73	62	1	G:A	M	T:T	24		7 (-3)	11	T:T	G:G
MBC74	61	0	G:A		C:C	22		10	11	C:C	G:C
MBC75	83	0	G:G		T:C	23		7 (-3)	11	T:C	G:G
MBC76	55	0	G:G		T:T	24		7 (-3)	7 (-3)	T:T	G:G
MBC77	50	1	G:G		T:C	23		7 (-3)	7 (-3)	T:C	G:G
MBC78	76	0	G:A		T:C					T:C	G:G

Fem Rels = Number of first-degree female relatives affected with breast cancer

BRCA2 Ex2 = BRCA2 exon 2 allele (G=wild-type, A=polymorphic)

BRCA2 Mut = Those found to carry BRCA2 mutation indicated by 'M'

ARG = Androgen Receptor Gene – Number of CAG repeats in Exon 1

ESR1 = Oestrogen Receptor Gene

7.5 Publications

Young IE, Kurian KM, Annink C, Kunkler IH, Anderson VA, Cohen BB, Hooper ML, Wyllie AH and Steel CM. A Polymorphism In The CYP17 Gene Is Associated With Male Breast Cancer. *Br J Cancer* 1999;81(1):141-43.

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Young IE, Kurian KM, MacKenzie MAF, Kunkler IH, Cohen BB, Hooper ML, Wyllie AH, Steel CM. The CAG Repeat Within The Androgen Receptor Gene In Male Breast Cancer Patients. *J Med Genet* 2000;37:139-140.

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Young IE, Kurian KM, MacKenzie MAF, Kunkler IH, Cohen BB, Hooper ML, Wyllie AH, Steel CM. A Polymorphic Tetranucleotide Repeat in the CYP19 Gene and Male Breast Cancer. *Br J Cancer* 2000;82(7):1247-48.

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BRCA1 and BRCA2 mutations in Scotland and Northern Ireland.

The Scottish/Northern Irish BRCA1/BRCA2 Consortium.

Br J Cancer 2003;88(8):1256-62.

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Short Communication

A polymorphism in the CYP17 gene is associated with male breast cancer

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Summary The CYP17 gene codes for the cytochrome P450c17 α enzyme that is involved in the synthesis of oestrogens. This case-control study from the South East of Scotland shows that a polymorphism of the CYP17 gene is associated with an increased risk of male breast cancer.

Keywords: CYP17 polymorphism; male breast cancer

Mutations of two tumour suppressor genes, BRCA1 and BRCA2, have been identified in some cases of familial and early onset breast cancer (Miki et al, 1994; Wooster et al, 1995). These mutations, however, account for no more than about 5% of total cases of female breast cancer. Male breast cancer is rare (approximately 1% of all cases of breast cancer) and therefore less is known about the genetic influences in its development. Male breast cancer has been linked to mutations of the BRCA2 gene in some cases. The frequency of mutations in the series studied varies widely, from 4% to 40% (Thorlacius et al, 1996; Friedman et al, 1997).

It has been suggested there may be other genetic factors that confer a relatively low absolute risk to the individual, but potentially could result in a substantial number of cases in the whole population (Feigelson et al, 1996). The CYP17 gene on chromosome 10 codes for the cytochrome P450c17 α enzyme that catalyses steroid 17 α -hydroxylase and 17,20-lyase activities (Picado-Leonard, 1987). It is therefore a key regulator in the synthesis of androgens and oestrogens from their steroid precursors (Brentano et al, 1990). A polymorphic T to C substitution has been described that creates an additional CCACC type promoter site 34 bp upstream from the site of initiation of translation (Carey et al, 1994). It is thought that the additional promoter site may increase the rate of transcription of the gene and thereby increase enzyme activity. Serum oestradiol levels are higher in women hetero- and homozygous for the C allele of the CYP17 gene (Feigelson et al, 1998). One study found that the C allele was associated with an increased risk of advanced breast cancer (Feigelson et al, 1997). A larger and more recent study, however, found no such association (Dunning et al, 1998). Male breast cancer is known to be associated with conditions, for example

Klinefelter's syndrome, that result in increased levels of serum oestrogens (Jackson et al, 1965). Mutation of the androgen receptor gene has also been reported in a few cases of male breast cancer (Wooster et al, 1992; Lobaccaro et al, 1993). This mutation results in a change in the androgen-oestrogen balance (low levels of androgens, increased levels of oestrogens). Given that the C allele of the CYP17 gene is associated with increased serum oestradiol levels, then it could also be implicated in the development of male breast cancer. The aim of this study was to test this hypothesis.

METHODS

Case and control population selection

Male cases were taken from a consecutive series of 76 male breast cancer patients treated in the South East of Scotland between 1974 and 1998. These patients ranged in age from 26 to 91 years at the time of diagnosis. Living patients were contacted through their General Practitioners. Initial contact was by telephone where possible. Peripheral blood samples (10 ml) were obtained from 24 living male breast cancer patients. Written informed consent was obtained in all cases. Archival wax-embedded tissue sections were obtained for 39 deceased patients. DNA previously extracted from a blood sample and stored was available for one of the deceased patients. Two patients declined to take part in the study. Archival specimens were not available in ten cases. Female cases were a consecutive group of 39 patients with early onset (age < 50 years) breast cancer referred to the Edinburgh Breast Unit, Western General Hospital, Edinburgh between 1987 and 1990.

Male and female control DNA samples were extracted from blood donations to the Edinburgh and South East Scotland Blood Transfusion service, from placental tissue and blood donations to the School of Biological and Medical Sciences, University of St Andrews, and from a small number of tonsillectomy specimens provided by the Department of ENT Surgery, City Hospital, Edinburgh. We believe that these samples are representative of the population within the South East Scotland region.

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Laboratory methods

DNA extraction was from whole blood by standard phenol-chloroform extraction. DNA extraction from wax-embedded tissue was from 10- μ m sections incubated at 55°C with a lysis buffer and proteinase K.

The following primers were designed for polymerase chain reaction (PCR): CYP17S-F, 5'-CAAAAGTCAAGGTGAA-GATCAG-3'; CYP17S-R, 5'-TAGGGTAAGCAGCAAGAGAG-3'. These generate PCR fragments 150 base pairs in length, including the polymorphic site. PCR reactions were performed in 50- μ l aliquots, each containing 1 \times buffer, 2 mM magnesium chloride, 100 μ M deoxynucleoside triphosphates, 100 pmol of each primer, 1 unit of *Taq* polymerase (Life TechnologiesTM) and approximately 100 ng DNA. The amplification was performed using an OmniGene thermal cycler (Hybaid, UK) under the following conditions: initial denaturation at 94°C for 3 min; amplification for 38 cycles, with denaturation at 94°C for 45 s, annealing at 56°C for 45 s and extension at 72°C for 45 s; final extension at 72°C for 10 min. The PCR products were digested with *Msp*A1 (Promega, Madison, WI, USA) for 90 min at 37°C. The products were separated by electrophoresis on a 4% agarose gel, staining with ethidium bromide and identified from photographs of the gel under ultraviolet illumination.

Data analysis

The association between the C allele of the CYP17 gene and breast cancer in the male and female subjects and controls was analysed using χ^2 tests.

RESULTS

The results showing the genotyping of the cases and controls are shown in Table 1. The results show that a C allele is found significantly more frequently in male breast cancer patients than in male controls ($\chi^2 = 4.308$, 1 d.f., $P = 0.038$). The odds ratio (OR) for the risk of a male with a C allele developing breast cancer is calculated

as 2.10 (95% confidence interval (CI) 1.04–4.27). There were no significant differences between female breast cancer patients and female controls ($\chi^2 = 1.895$, 1 d.f., $P = 0.169$; OR = 0.56, 95% CI 0.25–1.28). The distribution of alleles in both control groups is in Hardy-Weinberg equilibrium (Table 2).

DISCUSSION

To our knowledge, this is the first study that attempts to evaluate the possible role of a polymorphism of the CYP17 gene in the development of male breast cancer. We have found that the polymorphic T to C substitution within the CYP17 gene occurs significantly more frequently in male breast cancer patients than in male controls. These results are consistent with other reports that there may be a hormonal contribution to the development of male breast cancer (Jackson et al, 1965; Lobaccaro et al, 1993; Wooster et al, 1992). We have also studied a small group of female breast cancer patients from the same geographical region. We found no association between the presence of a C allele of the CYP17 gene and female breast cancer. The first study that looked at this polymorphism in female breast cancer patients (Feigelson et al, 1997), found a significant association only after sub-selecting those patients that presented with advanced disease. The second, and much larger, study (Dunning et al, 1998) did find a small increase in the risk of breast cancer associated with the C allele, but this increase was not statistically significant. It is possible that the T to C polymorphism in the CYP17 gene is associated with higher levels of serum oestrogens, but that because the endogenous oestrogen level is higher in females than in males, further increase does not have a significant effect. The association between an increase in the levels of serum oestrogens and the development of breast cancer may therefore be detectable only in males, or perhaps as previously suggested (Dunning et al, 1998), among post-menopausal females.

Further studies of this type are required, specifically to test for any association between the polymorphism of the CYP17 gene and levels of serum oestrogens in males.

Table 1 CYP17 genotype frequencies among breast cancer patients and controls

Genotype	Males		Females	
	Cases (n = 64)	Controls (n = 81)	Cases (n = 39)	Controls (n = 58)
TT	17 (26.6%)	35 (43.2%)	21 (53.8%)	23 (39.7%)
TC	42 (65.6%)	39 (48.1%)	13 (33.3%)	28 (48.3%)
CC	5 (7.8%)	7 (8.6%)	5 (12.8%)	7 (12.1%)
TC/CC	47 (73.4%)	46 (56.8%)	18 (46.2%)	35 (60.3%)

Table 2 CYP17 genotype frequencies in controls and expected frequencies under Hardy-Weinberg equilibrium

	Male controls		Female controls	
	Observed	Expected	Observed	Expected
TT	35	36.7	23	23.6
TC	39	35.7	28	26.8
CC	7	8.67	7	7.60
	($\chi^2 = 0.705$, 2 d.f., $P = 0.703$)		($\chi^2 = 0.116$, 2 d.f., $P = 0.943$)	

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Letters to the Editor

The CAG repeat within the androgen receptor gene in male breast cancer patients

EDITOR—Mutations of the *BRCA1* and *BRCA2* tumour suppressor genes have been identified in some cases of familial and early onset breast cancer.¹⁻³ Mutations of these genes, however, account for a relatively small proportion of the total cases of female breast cancer. Male breast cancer is a very rare disease, accounting for approximately 1% of all cases of breast cancer. Less is known about the genetic influences in its development. Male breast cancer has been linked to mutations of the *BRCA2* gene in some cases, with the frequency of mutations varying widely (from 4-40%) in those series studied.⁴

It has been suggested that there may be other genetic factors that confer a lower absolute risk to the person, but potentially could result in a substantial number of cases within a whole population.⁵ We have already shown that a polymorphism in the *CYP17* gene is associated with an increased risk of male breast cancer.⁶

A region within exon 1 of the gene coding for the androgen receptor (located on chromosome Xq11-12) is highly polymorphic and contains a variable number of CAG repeats. The variability of the number of these repeats between different ethnic populations in the USA has been studied.⁷ In vitro studies have shown that a relatively short CAG repeat sequence increases the level of transactivation of the androgen receptor.⁸ The androgen receptor itself binds dihydrotestosterone and therefore is one factor in the regulation of the growth of prostate cells. This may account for the finding that short CAG repeat sequences have been associated with a higher risk of developing prostate cancer.^{9,10} Abnormally long sequences of 40 repeats or more are found in patients with X linked spinal and bulbar muscular atrophy (Kennedy's disease).¹¹ This disease is associated with gynaecomastia and reduced fertility, suggestive of androgen insensitivity. Mutations of the androgen receptor gene may also result in reduced androgen receptor function and have been found in a few cases of male breast cancer.^{12,13}

The aim of this study was to investigate whether increased length of the CAG repeat sequence in the androgen receptor gene is associated with the development of male breast cancer.

The selection of male breast cancer cases and controls has previously been described.⁶ Ethical approval for the study was obtained through the Lothian Regional Ethics Committee.

DNA extraction was from whole blood by standard phenol/chloroform extraction. DNA extraction from wax embedded tissue was from 10 µm sections incubated at 55°C with a lysis buffer and proteinase K.

Using the published sequence,¹⁴ the following primers were designed (Primer Designer v1.1 ©1990 Educational Software): ARG-F 5'-TGCGCGAAGTGATCCAGA ACC-3', ARG-R 5'-CTCATCCAGGACAGGTAGCC-3'. These generate PCR fragments containing the CAG repeat sequence.

PCR reactions were performed in 50 µl aliquots, each containing 1 × PCR reaction buffer, 2 mmol/l MgCl₂, 5 µl

dimethyl sulphoxide, 200 µmol/l deoxynucleoside triphosphates, 20 pmol of each primer, 1 unit of *Taq* polymerase (Life Technologies™), and approximately 100 ng DNA. The amplification was performed using an OmniGene thermal cycler (Hybaid, UK) under the following conditions: initial denaturation at 94°C for three minutes; amplification for 38 cycles, with denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 45 seconds; final extension at 72°C for 10 minutes.

The products were denatured and then run on 6% polyacrylamide gels with a 10 bp DNA ladder. The products were then ranked in order of length. Three representative products were sent for automated sequencing (DNASHEF, Department of Haematology, Royal Infirmary of Edinburgh) to confirm the number of CAG repeats, and these were used as size standards. The products were then run again, with those thought to be of equal length adjacent to each other in order to check the accuracy of the original estimation of length. A second re-run was then performed to confirm the accuracy of the results.

The lengths of the PCR products obtained varied between 224 bp and 272 bp (corresponding to 14 CAG repeats and 30 CAG repeats, respectively). PCR was unsuccessful with DNA extracted from eight of the archival wax embedded tissue sections.

The distribution of alleles among male breast cancer patients and controls is shown in fig 1. The median number of CAG repeats in both groups was 23. There were no statistically significant differences between the two groups (Mann-Whitney test, $p=0.916$).

Three patients showed evidence of two different alleles indicating the presence of two X chromosomes (fig 2). One of these (MBC8) was recorded on the Edinburgh Cytogenetics Register with a diagnosis of Klinefelter's syndrome. The other two patients (MBC42 and MBC 62) had died, but there was no record of clinical suspicion of Klinefelter's syndrome in their hospital case notes. Neither fathered any children. The data were reanalysed following exclusion of these three cases. The median number of CAG repeats for the remaining 53 male breast

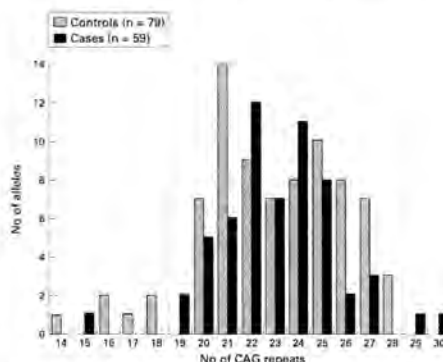


Figure 1 Distribution of CAG repeats in the androgen receptor gene among male breast cancer patients and controls.

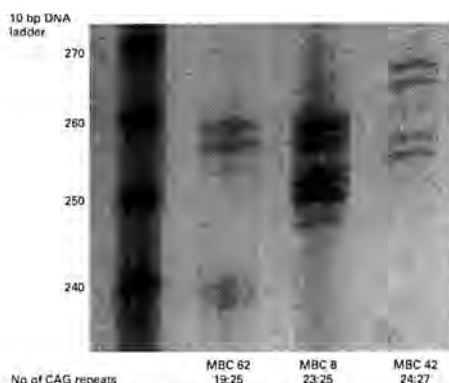


Figure 2 Genotyping of the three male breast cancer patients carrying two different alleles of the androgen receptor gene.

cancer patients was also 23. There was still no statistically significant difference between cases and controls ($p=0.765$).

We have not observed any overall difference between the median CAG repeat length of male breast cancer patients and controls. However, no males in the control group had alleles containing more than 28 CAG repeats, whereas two of the male breast cancer patients had alleles with 29 and 30 repeats respectively. Only one of the male breast cancer patients had an allele containing 18 repeats or less, compared to six of the controls. To our knowledge, the length of this CAG repeat has only been studied in one group of male breast cancer patients previously.¹⁸ There was found to be no significant difference between male breast cancer cases and controls. However, sequences of 30 repeats or more were found only among cases. Our results are consistent with these findings. In addition, it has been recently observed that women who are carriers of *BRCA1* mutations are at a significantly increased risk of breast cancer if they carry at least one androgen receptor gene allele with 28 or more CAG repeats.¹⁶ We believe that a relatively long CAG repeat sequence within the androgen receptor gene may be implicated in a few cases of male breast cancer. Conversely, a short CAG repeat sequence might offer a degree of protection against male breast cancer.

It is well recognised that Klinefelter's syndrome is associated with an increased risk of male breast cancer.¹⁷ One of the male breast cancer patients in our study was known to have had Klinefelter's syndrome. Our study of the androgen receptor gene has enabled us to identify a further two patients whom we suspect to have had Klinefelter's syndrome.

The findings presented in this study indicate that the CAG repeat sequence within the androgen receptor gene may, in some cases, be one useful molecular marker to identify males at increased risk of developing breast cancer.

Larger studies are required to define the importance of this CAG repeat in male breast cancer further. An international consortium has recently been set up and we have agreed to contribute our data to this.

There is also a GGC repeat sequence within exon 1 of the androgen receptor gene. This might be an interesting area for further study.

We thank the following: Mr R Morris and Dr S Bader for technical advice; Dr T Anderson, Dr A McGregor, Dr I Nawroz, Dr K Ramesar, and Dr A M Luffly for making available the archival wax embedded tissue sections; Miss G Kerr and the medical records staff in the Department of Clinical Oncology, Western General Hospital, Edinburgh; and staff in the Department of Blood Transfusion Medicine, Royal Infirmary of Edinburgh and in the Department of ENT Surgery, City Hospital, Edinburgh for providing some of the control samples. This work has been funded by grants from the Royal College of Surgeons of Edinburgh, the Sarah Percy Fund, the Melville Trust for the Care and Cure of Cancer, and the Robertson Trust.

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A polymorphic tetranucleotide repeat in the *CYP19* gene and male breast cancer

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Summary The *CYP19* gene codes for the aromatase enzyme that is involved in the synthesis of oestrogens. This case-control study examines the relationship between a tetranucleotide repeat sequence in the *CYP19* gene and the development of male breast cancer. No significant differences were found between male breast cancer cases and controls. © 2000 Cancer Research Campaign

Keywords: *CYP19*; male breast cancer

The *CYP19* gene (located on chromosome 15q21.1) codes for the aromatase enzyme that controls the rate-limiting step in the pathway of oestrogen synthesis from steroid precursors. It is known that an increased risk of breast cancer in males is associated with elevated serum oestrogen levels, for example in Klinefelter's syndrome (Jackson et al, 1965). The aromatase enzyme has been observed within the stromal cells in a greater proportion of male breast carcinomas than gynecomastia cases, suggesting that locally produced oestrogens may also have a significant role in the development of male breast cancer (Sasano et al, 1996). It is therefore possible that variation in expression of the *CYP19* gene could affect the risk of developing male breast cancer.

A polymorphic tetranucleotide (TTTA) repeat sequence is found in intron 5 of the *CYP19* gene, 79 nucleotides downstream from exon 4 (Means et al, 1989). This repeat sequence is relatively close to the exon-intron border and may therefore be involved in the determination of splicing sites (Kristensen et al, 1998). The aim of this study was to determine whether the development of male breast cancer is influenced by the length of this tetranucleotide repeat sequence in the *CYP19* gene.

METHODS

Case and control population selection

Male cases were taken from a consecutive series of 76 male breast cancer patients treated in the South East of Scotland between 1974 and 1998. Samples were available for DNA extraction in 64 of these cases. Control DNA samples were obtained from 79 healthy males representative of the South East Scotland population. Further details of male breast cancer cases and controls have

previously been described (Young et al, 1999). Ethical approval for the study was obtained through the Lothian Regional Ethics Committee.

Laboratory methods

DNA extraction was carried out from whole blood by standard phenol-chloroform extraction. DNA extraction from wax-embedded tissue was from 10-µm sections incubated at 55°C with a lysis buffer and proteinase K.

Primers as previously described (Polymeropoulos et al, 1991) were used for polymerase chain reaction (PCR): *CYP19-F*: 5'-GCAGGTACTTAGTTAGTAC-3'; *CYP19-R*: 5'-TTACAGT-GAGCCAAGGTCGT-3'. These generate PCR fragments that include the polymorphic site. PCR reactions were performed in 50-µl aliquots, each containing 1 × buffer, 2 mM magnesium chloride (MgCl₂), 200 µM deoxynucleoside triphosphates, 40 pmol of each primer, 1 unit of *Taq* polymerase (Life TechnologiesTM) and approximately 100 ng DNA. The amplification was performed using an OmniGene thermal cycler (Hybaid, UK) under the following conditions: initial denaturation at 94°C for 3 min; amplification for 38 cycles, with denaturation at 94°C for 45 s, annealing at 53°C for 45 s and extension at 72°C for 45 s; final extension at 72°C for 10 min.

The products were denatured and then run on 6% polyacrylamide gels with a 10 bp DNA ladder. The number of TTTA repeats in homozygous products were measured by cycle sequencing and these were used as size standards.

Data analysis

The distribution of alleles of the *CYP19* gene, comparing male breast cancer patients with controls, was analysed using the Mann-Whitney test. Odds ratios with 95% confidence intervals were calculated to show the risk of developing male breast cancer associated with each allele.

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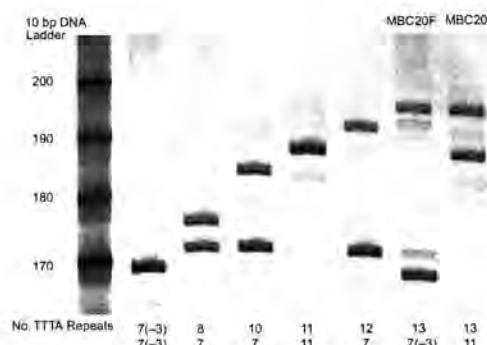


Figure 1 Examples of the seven different alleles of the *CYP19* gene found in male breast cancer patients and controls

Table 1 Distribution of alleles of the *CYP19* gene in male breast cancer patients and controls

Allele	PCR product length (bp)	Cases (n = 108)	Controls (n = 155)	Odds ratio & 95% confidence interval
(TTTA) ₇₋₃	168	35 (32.4%)	48 (30.4%)	1.10 (0.649–1.86)
(TTTA) ₇	171	20 (18.5%)	30 (19.0%)	0.970 (0.518–1.82)
(TTTA) ₈	175	11 (10.2%)	16 (10.1%)	1.01 (0.448–2.26)
(TTTA) ₁₀	183	1 (0.93%)	2 (1.3%)	0.729 (0.0653–8.14)
(TTTA) ₁₁	187	36 (33.3%)	57 (36.1%)	0.886 (0.529–1.48)
(TTTA) ₁₂	191	4 (3.7%)	5 (3.2%)	1.18 (0.309–4.49)
(TTTA) ₁₃	195	1 (0.93%)	0	–
Heterozygosity		79.6%	79.7%	

RESULTS

PCR was unsuccessful with ten of the DNA samples derived from archival wax-embedded tissue sections, giving a total of 54 case samples (108 alleles) analysed.

Seven different alleles of the *CYP19* gene were detected (Figure 1). The allele distribution of the *CYP19* gene in male breast cancer patients and controls is shown in Table 1. There were no significant differences in the distribution of alleles between cases and controls ($P = 0.838$). We have found two different alleles containing seven TTTA repeats (corresponding to PCR products of 168 bp and 171 bp in length). Cycle sequencing of these alleles revealed that the shorter allele, designated (TTTA)₇₋₃, had a 3-bp (TTC) deletion 50 bp upstream from the 5' end of the TTTA repeat sequence. One of the male breast cancer patients (MBC20) had an allele containing 13 repeats. A blood sample was obtained from his father (MBC20F), who had not had breast cancer. Analysis of DNA extracted from this showed the same (TTTA)₁₃ allele.

DISCUSSION

This is the first study that attempts to determine whether the development of male breast cancer is influenced by the length of the tetranucleotide repeat sequence within intron 5 of the *CYP19* gene. Two recent studies have determined the distribution of alleles among female breast cancer patients and controls. The first of these studies (Kristensen et al, 1998) found five different alleles

containing 7, 8, 9, 11 and 12 repeats. The allele containing 12 repeats was found significantly more frequently in female breast cancer patients than in controls. The second of the studies (Siegelmann-Danieli and Buetow, 1999) described eight different alleles by PCR product length. Details of TTTA repeat number were not given. Alleles of 168 bp and 171 bp in length were found, presumably corresponding to the two different alleles containing seven repeats found in our study, although the 3-bp deletion was not characterized. This deletion has, however, been described previously in a Japanese study (Kurosaki et al, 1997). From their study, Siegelmann-Danieli and Buetow (1999) conclude that the 171 bp allele represents a high-risk allele, whereas the 187 bp and 191 bp alleles (corresponding to 11 and 12 repeats respectively, from our data) are considered to confer low risk. These conclusions are contradictory to those drawn by Kristensen et al (1998).

Contrary to published (and mutually incompatible) findings in female breast cancer, our study found no significant differences in distribution of alleles between male breast cancer cases and controls. Patient MBC20, who has an allele with 13 repeats, was diagnosed with breast cancer at a very young age (26 years), but has no family history of cancer. This certainly represents a rare variant within the South East Scotland population, but is most likely to be an incidental finding.

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BRCA1 and BRCA2 mutations in Scotland and Northern Ireland

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BRCA1 and BRCA2 mutations have been identified in 107 families in Scotland and Northern Ireland. Ninety-seven of these families had been referred to regional cancer genetics centres and a further 10 were identified from a sequential series of male breast cancers treated in Edinburgh. Fifty-nine of the families had a mutation in BRCA1 and 46 in BRCA2. Two families had both. Family trees were extended and cancer diagnoses verified by means of the unusually complete and accessible Scottish and Northern Irish records. Ten specific recurring mutations (five in each gene) accounted for almost half of the total detected, and almost one-quarter were accounted for by just two (BRCA1 2800 delAA and BRCA2 6503 delTT). The prevalence of breast cancer is similar for BRCA1 and BRCA2 mutation families (average 3.7 and 3.6 per family), but the former have a much greater risk of ovarian cancer (average 1.5 and 0.6 per family, respectively). For breast cancer, age of onset tended to be younger in BRCA1 mutation carriers but, for ovarian cancer, there was no difference between BRCA1 and BRCA2 families in mean age at diagnosis. Mutations within the 5' two-thirds of BRCA1 carry a significantly higher relative risk of ovarian cancer and the same is true for mutations within the central portion of BRCA2 (the 'OCCR'). In the former case, this appears to be because of differences in absolute risk for both ovarian and breast cancer, while, in the latter, only ovarian cancer risk varies significantly. The findings confirm that founder mutations are present within the Scottish/Northern Irish population and have implications for the organisation of molecular screening services.

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Keywords: breast cancer; familial; BRCA1; BRCA2; Scotland; Northern Ireland

Worldwide variation in the distribution of BRCA1 and BRCA2 mutations is well recognised (Szabo and King, 1997). In several populations or ethnic groups, distinctive founder mutations form a sufficient proportion of the total to justify the adoption of specific molecular screening strategies (Levy-Lahad *et al.*, 1997; Peelen *et al.*, 1997; Thorlacius *et al.*, 1997; Moller *et al.*, 2001). In the UK, there has been only limited evidence to date of founder mutations (Gayther *et al.*, 1995, 1997; Eccles *et al.*, 1998; Lancaster *et al.*, 1998; Peto *et al.*, 1999), the most clear-cut example being BRCA1 2800 delAA, probably originating from the West of Scotland or Ireland (Liede *et al.*, 2000). Since the present day populations of Scotland and Northern Ireland remain relatively homogeneous, and distinct from those of other parts of the UK (Bodmer *et al.*, 1987; Liede *et al.*, 2000), we have collated information on all BRCA1 and BRCA2 mutations recorded among families attending breast cancer genetics clinics in Aberdeen, Belfast, Dundee, Edinburgh or Glasgow. Additional data are included from a sequential series of male breast cancers presenting in South-East Scotland (Young *et al.*, 2000). Findings are compared with those reported from England, Wales and elsewhere.

In both Scotland and Northern Ireland, families have tended to be large, at least until the current generation. They are also, typically, close-knit, maintaining contact even with members who have emigrated to distant parts of the world. Records of births, marriages and deaths are unusually complete and accessible

(Collyer and DeMey, 1987) and the Scottish Cancer Registry, in particular, is recognised as among the best in the world (Kemp *et al.*, 1985). For all these reasons, data on Scottish and Northern Irish families with genetic disorders can be extensive and accurate. The present data set, based on over 100 mutation-bearing families, is therefore of value for addressing questions about the clinical implications of BRCA1 and BRCA2 mutations.

PATIENTS, FAMILIES AND METHODS

The study was approved by the Medical Research Ethics Committee for each of the regions in which clinical genetics services are based. The five centres mentioned above provide comprehensive National Health Service (NHS) clinical services for familial cancers, covering the entire population. They have collaborated closely in the design and development of these services for some 10 years and follow common policies on eligibility for regular surveillance by clinical/mammographic and ovarian screening (Haites *et al.*, 2000). Molecular testing for BRCA1 or BRCA2 mutations was available only on a research basis from 1994 until 2001, when NHS funding was provided to establish a laboratory service accessible to all high-risk families. The majority of mutations included in this report were identified in one of the Scottish or Northern Irish research centres but some were found in family members tested at the Institute of Cancer Research, London (Dr Simon Gayther, Prof Michael Stratton), the University of Lund (Dr Ake Borg) or in centres in the USA, Australia, Iceland and the Netherlands. Mutation-detection methods therefore varied but, in general, they have been based on PCR amplification of gene fragments, covering most of the coding regions and splice sites,

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followed by SSCP or heteroduplex analysis on gels (Gayther *et al.*, 1995, 1996) or, more recently, by DHPLC (Wagner *et al.*, 1999). Protein truncation assay (Hogervorst *et al.*, 1995) has been applied on a limited scale and 150 samples from 'high-risk' families were screened specifically for the 6 kb duplication in BRCA1 exon 13, with one positive result (Mazoyer *et al.*, 2000). Only some 20 'high-risk' Scottish families have been included in a preliminary screen for large deletions in BRCA1. None has yet been found. Some centres have concentrated on screening those regions of both genes where mutations have already been identified in our population. This is likely to increase the apparent frequency of recurrent mutations but it is difficult to quantify the effect. The bulk of the screening was undertaken without this bias and, where priority was given to examination of particular PCR fragments, these, in total, represent a significant proportion of the entire coding regions of BRCA1 and BRCA2, so that nonrecurrent mutations could be (indeed were) detected in the process. All mutations have been confirmed by direct sequencing and reconfirmed on a second blood sample from the index case before offering counselling and access to genetic testing for at-risk adult members of the family.

Family trees have been constructed, initially from information provided by families themselves. In most instances, they have filled out detailed family history forms, issued on referral to a genetics clinic. This normally entails a collaborative effort on the part of several family members, across the generations. Verification and extension of pedigrees can be undertaken by consulting public registration records (Collyer and DeMey, 1987) which, in comprehensive form, date from 1855. This facility, supported by a professional medical genealogist, is used selectively but when a germline mutation is detected, record tracing becomes a priority, since the process can establish links between two or more families already known to the clinics.

All practicable efforts are made to verify reported cancer diagnoses in the family, particularly if it seems probable that the pattern of disease is attributable to a high penetrance mutation. Confirmation can be obtained, in the case of deceased subjects, by consulting the Scottish or Northern Irish cancer registries (good data are available from mid-1960s onwards). For living patients, permission is sought to check relevant details from hospital records. In some instances, particularly for cancers that occurred over 40 years ago, supporting evidence can be obtained only from death certificates and, on occasions, there is no means of verification. A judgement then has to be made, on the basis of the family's own knowledge, whether or not to record a given relative as being affected by breast, ovarian or other cancers. When a mutation has been identified in one member of a given family, blood samples or tissue blocks are sought from affected relatives, to establish their mutation status. The process of tracing the distribution of the mutant allele within each family is still ongoing. In a number of cases, affected relatives have been shown not to carry the 'family mutation'. These individuals are usually then reclassified as having sporadic cancer (with correspondingly reduced risk estimates for their descendants) but in two families a second mutation has been found and, in a few more, the pattern of cancers on both sides of the pedigree would be consistent with the presence of a second, still unidentified, mutation.

In the main, families selected for molecular testing met the published criteria (Hailes *et al.*, 2000) for, at least, 'moderate risk' (i.e. two close relatives with early onset breast or ovarian cancer) and most met the more stringent criteria defining 'high risk'. Two exceptions were a single positive result from a consecutive series of women diagnosed with breast cancer under age 50 and one patient who had only a single relative with breast cancer, but an unusual familial cluster of early onset prostate, stomach and laryngeal cancers. She herself might have been discharged but was found to have breast cancer on her first clinic visit and a BRCA2 mutation was subsequently demonstrated. Twelve BRCA2 mutations have

been identified among DNA samples from a consecutive series of 64 male breast cancers treated in Edinburgh since 1975. These samples were analysed, in the first instance, without reference to family history (Young *et al.*, 2000). Most of the patients are deceased and construction of complete family trees is still proceeding, with the cooperation of their living relatives. However, two of them belong to families already registered with the regional cancer genetics clinic (because a cluster of cases among relatives had been recognised). In addition to the 12 from the consecutive series, a further 10 instances of male breast cancer have been recorded among the BRCA2 mutation-positive families in the present report. Two of these families included more than one affected male (three and two cases each).

RESULTS

One hundred and nine mutations (61 in BRCA1, 48 in BRCA2) have been identified in 107 families. The vast majority cause truncation of the protein product (89 frame-shifts, six nonsense substitutions, eight splice site disruptions). One is a 6 kb duplication of BRCA1 exon 13 and there are two instances of an intronic deletion believed to affect splicing (BRCA2 9877-67 *del4*). Only three missense substitutions are included. Two of these are recognised as pathogenic by the Breast Cancer Information Core (BIC) database and the third, BRCA1 5242C>A, although still listed as an unclassified variant, has been recorded 13 times in multicase breast cancer families.

Several mutations have been identified in more than one family each. Two are particularly frequent, BRCA1 2800 *delAA* and BRCA2 6503 *delTT*. The former has already been identified as a Scottish/Irish founder mutation (Liede *et al.*, 2000), while the latter is also a recognised founder mutation, distributed throughout Europe and the USA (Mazoyer *et al.*, 1996; Szabo and King, 1997; BIC database). In contrast to BRCA1 2800 *delAA*, the BRCA2 6503 *delTT* mutation is relatively frequent among families attending the Aberdeen and Dundee clinics (representing the North and East of Scotland), which may be in keeping with Scandinavian origin (Table 1).

Eight further mutations, each detected at least three times, account for 27 families. Two of the three recognised Ashkenazi Jewish mutations (Levy-Lahad *et al.*, 1997) – BRCA1 185 *delAG* and BRCA1 5382 *insC* – are among these eight; the third, BRCA2 6174 *delT*, was found on two occasions. The overall distribution of mutations is set out in Figure 1.

In assessing the numbers of breast and ovarian cancers recorded in mutation-positive families, two families have been excluded (both with BRCA2 mutations). One was identified through the consecutive series of male breast cancers and no information has yet been obtained about relatives. The other has been counted as a BRCA1 mutation family, although a single BRCA2 mutation-

Table 1 Geographical distribution of BRCA1 and BRCA2 founder mutations

	BRCA1 2800 <i>delAA</i>	BRCA2 6503 <i>delTT</i>
Aberdeen	0	4
Dundee	1	4
Edinburgh	4	1
Glasgow	7	5
Belfast	2	0

Numbers of families with founder mutations BRCA1 2800 *delAA* or BRCA2 6503 *delTT* attending each of the five clinics in Scotland/Northern Ireland. Some families, with branches in different parts of the country, are recorded as attending more than one clinic. Clinics are listed in geographical sequence from North-East (Aberdeen) to South-West (Belfast).

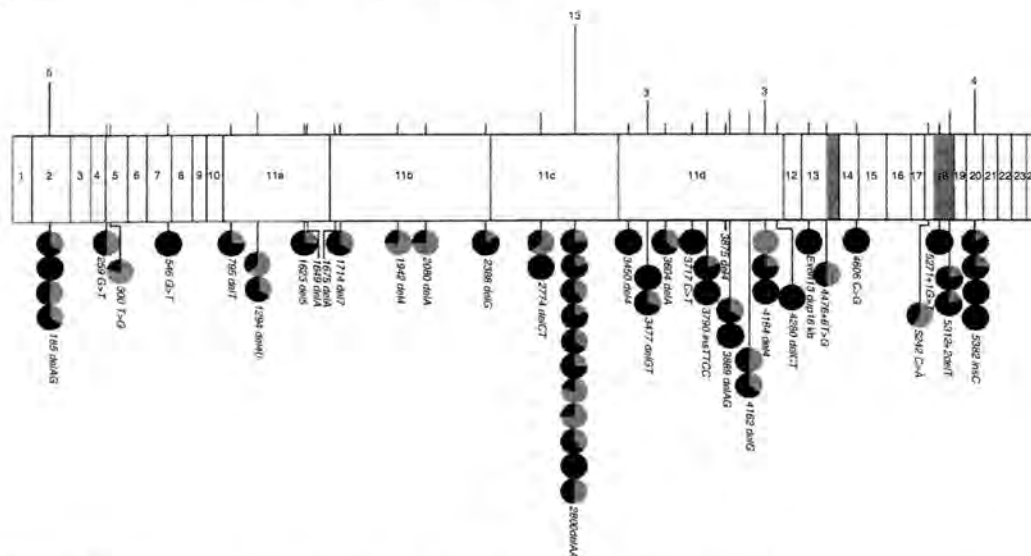


Figure 1 Diagrammatic representation of BRCA1 exons (plus intronic regions with pathogenic mutations, shown shaded). Vertical lines above the diagram represent all mutations detected, with heights proportional to numbers of cases. Actual numbers are given for most frequent mutations. Circles below the diagram represent families with at least three breast or ovarian cancers attributed to each mutation. The darker segment of each circle represents the proportion of breast cancers and the paler segment the proportion of ovarian cancers.

carrier has been identified in one branch of the pedigree. That branch has not yet been investigated in detail. For the other family with two mutations (BRCA1 and BRCA2), it has been possible to identify the separate routes of inheritance and hence to calculate the numbers of breast and ovarian cancers attributable to each mutation. For all families, where an affected individual has been found not to carry the BRCA1 or BRCA2 mutation, that tumour has not been included in the calculations and, of course, only tumours occurring in blood relatives of mutation-positive family members are counted. Overall, comparable effort has been expended on gathering and verification of data on BRCA1 and BRCA2 mutation families. As shown in Table 2, the average number of breast cancers recorded per family is virtually the same for both mutations, but there are two and a half times as many ovarian cancers in BRCA1 families. The ratio of breast to ovarian cancers is 2.39:1 for BRCA1 families and 6.18:1 for BRCA2 families, a difference which is significant at the 0.01% level by χ^2 analysis. This is consistent with data from the Breast Cancer Linkage Consortium (Ford *et al*, 1998) and other sources (Risch *et al*, 2001; Welch and King, 2001).

The mean age at diagnosis of breast cancer in BRCA1 mutation carriers was 42.6 years (95% CI 40.5–44.8). For BRCA2 mutation carriers (females only), it was 46.5 years (95% CI 43.6–49.4). Half of those with BRCA1 mutations were diagnosed at or below age 40, compared with just over one-quarter of those with BRCA2 mutations. For affected males (all with BRCA2 mutations), mean age at diagnosis of breast cancer was 59.4 years (95% CI 51.9–67.0). In keeping with the younger age at onset, 56% of BRCA1 families included at least one instance of bilateral disease, compared to 28% of BRCA2 families. (In the latter set, there was one male with bilateral breast cancers, onsets separated by 22 years.) Furthermore, in 36% of BRCA1 families, but fewer than 10% of BRCA2 families, there were two or more examples of

bilaterality. Individual women with both breast and ovarian cancer were recorded in 36% of BRCA1 families, with up to three instances per family, whereas they occurred in only 10% of BRCA2 families and no family included more than one case. Ages of onset for ovarian cancer did not differ between families with BRCA1 or BRCA2 mutations (49.7 years, 95% CI 46.9–52.4 and 49.8 years, 95% CI 43.0–56.6 years, respectively). Over 90% of ovarian cancers, in both sets of families, were diagnosed at age 40 or older and half of the remainder were diagnosed at age 39.

A rather more contentious issue is the effect of mutation position, within either gene, on the relative risk of breast or ovarian cancer (Gayther *et al*, 1995; Iau *et al*, 2001; Thompson *et al*, 2001). For BRCA1, there is evidence that mutations towards the 3' end are less likely to be associated with ovarian cancer (Gayther *et al*, 1995). We have subdivided our BRCA1 families as shown in Tables 3A, B, and confirm that the ratio of breast to ovarian cancers is strongly influenced by mutation site. Interestingly, our data suggest that this may result from both an increase in breast

Table 2 Breast and ovarian cancers recorded in BRCA1 and BRCA2 mutation-bearing families

	BRCA1 mutation +ve	BRCA2 mutation +ve
No. of families	61	46
No. of breast cancers	225	167
No. of ovarian cancers	94 ^a	27 ^b
Av. breast cancers/family	3.7	3.6
Av. ovarian cancers/fam	1.5	0.6
Ratio CaBr/CaOv	2.39:1	6.19:1

^aIncludes one primary peritoneal carcinoma. ^bIncludes one primary carcinoma of fallopian tube.

*Difference in risks of breast cancer $P = 0.001$ (by χ^2). ^bDifference in relative risks of breast vs ovarian cancer, $P = 0.0002$ (by χ^2). ^cDifference in risks of ovarian cancer $P < 0.02$ (by χ^2). ^dDifference in relative risks of breast vs ovarian cancer $P < 0.005$ (by χ^2).

cancer risk and a reduction in ovarian cancer risk when the mutation occurs 3' of exon 11.

For BRCA2, it has been proposed that mutations within the central region of the gene, termed the ovarian cancer cluster region ('OCCR'), are particularly associated with ovarian cancers

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The proportion of BRCA2 mutation-bearing families in the present series (44%) is higher than that has been estimated indirectly from earlier (mainly much smaller) studies from the UK (Szabo and King, 1997; Lancaster *et al.*, 1998). However, it is consistent with the prediction of roughly equal prevalence of BRCA1 and BRCA2 mutations, based on a survey of very early onset breast cancers (Peto *et al.*, 1999). The authors of that survey noted that several UK studies have concentrated on multicase families in which ovarian cancer was prominent, thus overestimating the relative frequency of BRCA1 mutations. The International Breast Cancer Linkage Consortium data show that, among families with multiple cases of breast cancer but no known ovarian cancers, BRCA1 and BRCA2 mutations are almost equally represented (Ford *et al.*, 1998). In the present series, there was no particular emphasis on ovarian cancer in selecting families for mutation testing but, of course, the clustering of ovarian with breast cancer in a family adds considerably to the prior probability of a mutation being present, as also does the occurrence of very early onset breast cancer. Account is taken of both these facts in the Scottish NHS guidelines



for management of familial cancers (Haites *et al.*, 2000), hence favouring ascertainment of BRCA1 mutation-bearing families. In most centres, facilities for BRCA1 mutation testing became available at least a year before those for BRCA2 so that, in consecutive series, there is some bias in favour of detecting BRCA1 mutations. This applies to the present data set, although the effect is probably not great and should diminish with time. On the other hand, our inclusion of 10 BRCA2 mutations detected only through a survey of male breast cancers obviously introduces a bias in the opposite direction. Nevertheless, it appears that in Scotland and Northern Ireland, the relative prevalence of BRCA2 mutations may be higher than in some other regions of Europe such as Norway (Møller *et al.*, 2001), Sweden (Hakansson *et al.*, 1997), Belgium (Claes *et al.*, 1999) or France (Serova-Sinilnikova *et al.*, 1997; Stoppe-Lyonnet *et al.*, 1997).

Our findings do not permit any calculation of the absolute prevalence of BRCA1 or BRCA2 mutations in the Scottish and Northern Irish populations but they add to our knowledge of the distribution of specific mutations. Allowing for some bias in ascertainment, as discussed above, almost one-quarter of the families are accounted for by just two (BRCA1 2800 delAA and BRCA2 6503 delTT) and almost half by the 10 mutations that were found in at least three families each. Two of these are the well-recognised 'Ashkenazi Jewish' BRCA1 mutations (185 delAG and 5382 insC), although at least one of the affected families is unaware of any Ashkenazi Jewish ancestry. The other recurring mutations are BRCA1 3477 delGT and 4184 delA, BRCA2 1418 insA, 5445 delT, 7165 + 2 delT, 7830 del16. Most of these have been recorded many times in the BIC database and/or the Human Gene Mutation Database. Our earlier speculation (Liede *et al.*, 2000) that BRCA1 2800 delAA may have originated in West-Central Scotland or in Ireland seems to be supported by the finding of three further cases, one from Glasgow and two from Belfast. BRCA2 6503 delTT has been found elsewhere in the UK as well as in Dutch, Swedish, Danish and Belgian families (BIC database). It was the most frequently recurring BRCA2 mutation (four examples) among 25 recorded by Gayther *et al.* (1997) from 'the United Kingdom and Eire' but no further breakdown by geographical region was provided. BRCA1 4184 delA was identified as a recurring mutation in that survey and also in separate reports of families from Wales and the South of England (Lancaster *et al.*, 1998; Eccles *et al.*, 1998). In the last-mentioned study, BRCA1 3875 was found five times in a total series of 17 mutations. It is entirely possible that further examples may be found of founder mutations associated with specific populations concentrated in particular regions of the UK but larger studies will be required. Scotland and Northern Ireland offer special advantages for this kind of analysis because of the relative population stability, the existence of an NHS-funded cancer genetics network and the excellence of genealogical and health-related records.

The proportion of all breast or breast/ovarian cancer families in our population attributable to recurring BRCA1 or BRCA2 mutations appears to be lower than in some other countries such as Iceland (Thorlacius *et al.*, 1997), Israel (Levy-Lahad *et al.*, 1997) or Norway (Møller *et al.*, 2001), comparable to that in the Netherlands (Peelen *et al.*, 1997; Claes *et al.*, 1999), but much higher than in others, such as Italy (Szabo and King, 1997; Cippolini *et al.*, 1999), and sufficient to have practical implications for the formal organisation of molecular screening. A first-stage analysis, covering the 10 most common BRCA1 and BRCA2 mutations, using multiplex PCR/DHPLC is rapid and relatively inexpensive. It can be offered to substantial numbers of families, then, if a negative result is obtained, more stringent risk criteria can be applied for complete analysis of the genes. This policy is already identifying further mutation-positive families (not included in the present report) among those who would not currently be eligible for full-scale molecular screening in our diagnostic laboratories (Haites *et al.*, 2000). The occurrence of

identical mutations in many families also provides opportunities for investigation of genetic and environmental factors that may modify penetrance or expression of the underlying mutation. This principle has been applied, for example, to identify an interaction between BRCA2 mutations and a polymorphism of RAD51 (Levy-Lahad *et al.*, 2001; Wang *et al.*, 2001). Among the families recorded here is one with seven cases of ovarian cancer over four generations, but no breast cancers. No other family with the same mutation, from the present series or from the BIC database, shows a comparable distribution of tumours and further research is warranted to establish whether this is merely a chance finding or a clue to an additional causal factor.

The influence of mutation site on relative risk of breast or ovarian cancer has been a controversial issue. For BRCA1, two studies published in 1995 indicated that the relative risk of ovarian cancer was substantially higher for mutations occurring in the 5' two-thirds of the gene (Gayther *et al.*, 1995; Shattuck-Eidens *et al.*, 1995) but this was not confirmed in three other reports (Serova *et al.*, 1996; Couch *et al.*, 1997; Frank *et al.*, 1998) and is not apparent in a fourth (Eccles *et al.*, 1998), where the data are presented but not discussed in detail. Taking the 3' end of exon 11 as the potential 'change point', we found a highly significant difference in the relative proportions of breast and ovarian cancers 'upstream' and 'downstream' of this position. Furthermore, the difference seems to arise both from an increased risk of breast cancer and a reduced risk of ovarian cancer for mutations in the 3' third of the gene, although the difference in numbers of breast cancers per family does not achieve statistical significance on its own ($P < 0.1$).

For BRCA2, an 'Ovarian Cancer Cluster Region' in the middle third of the gene was proposed by Gayther *et al.* (1997). An international collaborative study (Neuhausen *et al.*, 1998) provided some supporting evidence which, however, failed to reach statistical significance. In a more recent analysis of 164 BRCA2 mutation-bearing families (Thompson *et al.*, 2001), 23.8% of tumours were ovarian (and 76.2% breast) among families with OCCR mutations: for families with mutations 5' and 3' of the OCCR, 8.8 and 9.6%, respectively, were ovarian cancers. Our own findings are similar but, whereas Thompson and colleagues found that a reduced absolute risk of breast cancer for OCCR mutations contributes substantially to the effect, in our series, the number of breast cancers per family is actually higher for OCCR vs non-OCCR mutations, and the entire increase in relative risk of ovarian cancer is attributable to a 2.9-fold higher rate of ovarian cancers in the former group. It will be important to determine which of these interpretations is correct in order to generate testable biological hypotheses to explain the findings.

The validity of our analysis depends on completeness of ascertainment of families and accuracy of breast and ovarian cancer diagnoses. There are obviously limitations on both counts but the large average family size, the accessibility of confirmatory records and the uniformity of our approach to extension and verification of family data allow some confidence in our data. Excluding nine families ascertained only through a series of male breast cancers (one such family was already excluded for lack of information) greatly reduces the statistical power of our analysis. It happens that all of these nine families carried mutations outside the OCCR and that none included any case of ovarian cancer. However, there is no indication that these are particular characteristics of BRCA2 mutations associated with male breast cancer. Six of the families with mutations inside the OCCR included affected males (two with more than one male case each) and two of these also included ovarian cancers.

Whether the associations between mutation position and phenotype are sufficiently strong to influence genetic counselling and management of affected families remains a moot point. Individual families, where the distribution of cancers seems to depart from the general rules, are not rare and, for the present, it

may be wise to take at least as much account of the previous history of the family in question as of the statistical findings from large-scale surveys.

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ELECTRONIC-DATABASE INFORMATION

(BIC) Breast Cancer Information Core Database.
Human Gene Mutation Database. <http://www.uwcm.ac.uk/wcm/mg/hgmd0.html>

APPENDIX 1.

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Dr. K. Kurian studied the histological sections.

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7.7 Declaration

This thesis was written entirely by myself.

All grant applications (with the exception of the grant from the Robertson Trust) were written by myself as principal investigator.

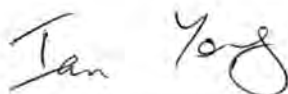
I collected all of the samples for DNA extraction and interviewed all of the living patients myself.

I performed more than half of the total laboratory work.

Dr. M. MacKenzie performed approximately half of the BRCA2 screening, working with me for ten months.

Miss. C. Annink performed a small amount of the BRCA2 screening and analysed some of the controls for the CYP17 polymorphism. This was while working as a research student on elective, under my supervision for six months.

Miss. J. Back performed some of the initial sequencing, mainly looking at the BRCA2 exon 2 polymorphism, for a period of two months.



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October 2003

Ian Young

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